EFFECT OF INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN-4 GENE THERAPY ON ADENOCARCINOMA OF THE COLON

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Abstract

Insulin-like growth factors induce the proliferation of transformed cells. IGF binding proteins (IGFBP) are involved in their local tissue regulation. In this project, the effects of early and late administration of IGFBP-4 gene were assessed on colon cancer model *in vivo*. Nude mice were subcutaneously inoculated with HT-29 colon adenocarcinoma cells. In the early gene transfer model, IGFBP-4 gene was administered along with cancer cells. In the late gene transfer model, cancer was induced first and IGFBP-4 gene was administered when the tumour became visible (one week after inoculation). Animals received either mammalian expression vector containing IGFBP-4 cDNA, or vector alone, or PBS as peritumoral injection. Tumour size was measured at different time periods during the experiment. After three to four weeks of IGFBP-4 induction, the mice were sacrificed and tumour tissues were collected for further examinations. Tumour proliferative activity was determined by counting mitotic cells. Tumour apoptosis was investigated by TUNEL assay and electron microscopy. Results showed that tumour tissues had large necrotic areas, significantly increased numbers of apoptotic cells, and decreased cells undergoing mitosis following treatment with IGFBP-4 gene, in both early and late gene transfer model. Despite increased apoptosis and decreased mitosis in IGFBP-4 treated tumours, tumour volume was not significantly altered, possibly due to cellular debris filling the centre of tumours. There was an increase in Bax protein levels after IGFBP-4 gene therapy in both models. When IGFBP-4 gene was administered late, tumours showed higher expression of IGFBP-4 protein levels as well as IGF-IR levels when compared with controls. However, in the early gene transfer model, when IGFBP-4 gene was administered along with cancer cells, the tumours did not show such an increase in IGFBP-4 levels compared with controls. Although the tumours of the late group showed a reduction in Bcl-2 protein levels, the early gene transfer model did not show a similar reduction. In fact the Bcl-2 was elevated after IGFBP-4 gene therapy.

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Abbreviations

ALS acid-labile subunit

Bp base pair

BP-4 IGFBP-4

cDNA complementary deoxyribonucleic acid

Cox-2 cyclo oxygenase

DNA deoxyribonucleicacid

E. coli Escherichia coli

EGTM Early gene transfer model

FCS foetal calf serum

IGF-I, -II insulin-like growth factor-I, -II

IGF-IR type I IGF receptor

IGF-IIR type II IGF receptor

IGFBPs IGF binding proteins

IGFBP-rPs IGFBP-related proteins

KDa kilo Dalton

Kb kilo base

LGTM Late gene transfer model

LOI Loss of inhibition

mAmp milliampere

mg milligram

ml millilitre

mm millimetre

mM milli molar

NSAIDS Non steroidal anti-inflammatory drugs

PBS phosphate buffered saline

PCR polymerase chain reaction

Rpm revolution per minute

RT-PCR reverse transcription polymerase chain reaction

μg micro gram

μl micro litre

Chapter 1 Introduction

The incidence of colorectal cancer has been increasing rapidly since 1975, with about 300,000 new cases and 200,000 deaths in Europe and the USA each year [Midgley and Kerr, 1999;Boyle and Langman, 2000]. It is the second commonest cause of death from any cancer in men in the European Union [Boyle and Langman, 2000]. Long-term survival of colorectal cancer is related to the stage of the disease. If detected early, it may be curable by surgery [Dorudi et al., 2002]. But once metastases develop the prognosis becomes poor. At least 50% of patients with colorectal cancer develop recurrence or metastases during their illness [Midgley and Kerr, 2000]. Though various combinations of surgery, radiotherapy and chemotherapy are used for advanced cancer, the most effective approach is yet to be discovered.

Several growth factors, including transforming growth factor-β, epidermal growth factor, insulin and insulin-like growth factors (IGFs) have been implicated in the development of colon cancer [Schmelz et al., 2007;Coban et al., 2007]. In the last decade, the role of IGFs in cancer has been increasingly recognised. The IGFs are peptides structurally related to insulin that regulate the proliferation of several mammalian cells, including epithelial cells of breast, prostate, colon and lung [Pollak, 2000]. Abnormal expression of the IGFs, their receptors and binding proteins have been linked to several cancers including colorectal cancer [Mishra et al., 1998;Khandwala et al., 2000]. Correction of the malfunctioning genes in the form of gene therapy may prevent or cure cancers.

1.1 Gene therapy in colorectal cancer

This chapter will provide a brief outline about gene therapy and the rationale behind its use with relevance to colorectal cancer.

1.1.1 Gene therapy

Genes are building blocks of deoxy ribonucleic acid (DNA) which are found inside cells. Genes may encode enzymes or proteins and are ultimately responsible for the production of them. Potentially any change in gene expression, such as over-expression, deletion and malfunction could result in cancer. There are approximately 50,000 to 100,000 genes in the human body. Different genes are active in different phases of cell cycle. Gene therapy involves the introduction of genetic materials, which are nucleic acids, into the cells. The genetic material may be deoxy ribonucleic acid or ribonucleic acid (RNA), which may help to replace or correct the malfunction due to a single gene. Gene therapy can also be performed either to trigger an immune response or to produce a therapeutic substance.

In general, there are two ways of transferring genes to mammalian cells. The first way is transferring an exogenous gene directly into cells by local or systemic administration. The second way is culturing cells from a patient and transfecting them with the required gene *in vitro* and re-introducing them into the patient. It was Dr. W. French Anderson who performed the first government-approved human gene transfer for a 4-year-old girl with severe combined immunodeficiency in 1990 in the USA. Following the procedure the patient was not cured but her condition improved and she acquired resistance to frequent cold. It stimulated interest in the scientific

community about gene therapies. Currently several gene therapy trials are being carried out throughout the world especially for cancer.

1.1.2 Introduction of genes into the mammalian cell

Genes have to be transferred into a cell by means of a vector to enable them to function. The vectors that are used can be classified into viral and non-viral.

Viral vectors

Viral vectors can be used to introduce a DNA into eukaryotic cells. These are more powerful than the non-viral transfection system. A variety of viral vectors have been employed to deliver genes to cells to provide either transient (e.g. adenovirus, vaccinia virus) or permanent (e.g. retrovirus, adeno-associated virus) transgene expression, and each approach has its own advantages and disadvantages [Young et al., 2006]. Complications which may occur during virally mediated gene therapy include virus dissemination [Wang et al., 2003] causing toxic shock, leukaemia [Puck and Malech, 2006], immune reactions against the vectors causing failure of gene therapy, and inappropriate insertion of vectors and transgenes that may cause mutations leading to cancer.

Retrovirus

The first human gene therapy trial was performed by Rosenberg in 1989 using retrovirus [Aebersold et al., 1990]. He introduced genes encoding resistance to neomycin into human lymphocytes and infused them into 5 patients with advanced melanoma. Only one person exhibited remission. The main drawbacks of retrovirus are lack of tissue specificity, low production titres and the need for cell replication for infectivity. A recent animal experiment showed a significant reduction of multiple tumour foci in the liver when cytosine deaminase (CD) gene was transferred

using retrovirus and prodrug 5-fluorocytosine was infused into the portal circulation [Hiraoka et al., 2007] .

Adenovirus

Adenovirus is responsible for the common cold. Replication-deficient recombinant adenoviral vectors are predominantly used for colon cancer gene therapy, because they can be produced at a high titre and they readily infect a number of different cell types [Zwacka and Dunlop, 1998]. They also have a tropism for the liver and can infect both dividing and quiescent cells [Li et al., 2007]. Therefore, these vectors are also useful for gene therapy involving the liver.

Other viruses

Herpes simplex virus, which causes cold sores, can infect a wide range of dividing cells[Nakano et al., 2005]. Other viruses which may be useful as a vector include vaccinia virus, lenti virus and heamagglutinin virus of Japan.

Non-viral vectors

There are at least five non-viral methods of introducing DNA into the mammalian cells. They are calcium phosphate transfection [Maitra, 2005], DEAE-dextran transfection, electroporation [Cemazar et al., 2006], liposome mediated transfection [Karmali and Chaudhuri, 2006] and plasmid mediated gene transfer. The first two procedures produce a chemical environment that results in DNA attaching to the cell surface. The DNA is then endocytosed by uncharacterized pathways.

Electroporation uses an electric field to open up pores in the cell. The DNA presumably diffuses into the cell through the pores. So this technique can be used in any cell type. Electrogene therapy is an *in vivo* application of electroporation where

naked plasmid DNA is injected and electric pulses are delivered directly to the tissue [Cemazar et al., 2006]. However, the efficiency of this method *in vivo* is still lower than virally mediated gene transfer. Nevertheless, electrically-assisted nucleic-acid delivery holds a great potential for the clinical application due to the lack of immunogenicity, easiness of the preparation of large quantities of endotoxin free plasmid DNA, reproducibility and the development of electro-pulsators approved for clinical use [Cemazar et al., 2006].

In liposome-mediated transfection, liposomes containing cationic and neutral lipids mediate the transfection of DNA. The advantages of this method include: a) it is easy to prepare (b) ability to inject large lipid:DNA complexes; and (c) low immunogenic response [Karmali and Chaudhuri, 2006]. The mechanism is poorly understood.

Plasmids can also be used for transferring DNAs. They are extra chromosomal DNA molecules which are self replicating. Escherichia coli (E.Coli) bacteria carry plasmids which offer resistance against antibiotics, heavy metals and obscure bacteriophages. Replication of these plasmids may or may not require plasmid-coded proteins and may or may not be synchronised with cell cycle. Some of these plasmids can be freely transferred from one bacteria to another. Artificial plasmids have been constructed in laboratories since 1970, with fragments of DNA and naturally occurring plasmids. All these plasmids have 3 common features. They all have a replicator, a selectable marker and a cloning site. A replicator is a stretch of DNA that contains the site at which DNA replication begins. A selectable marker is an ab gene encoding resistance to some antibiotics. The cloning site is a restriction endonuclease cleavage site into which foreign DNA can be introduced without

interfering with the plasmids' ability to replicate. Uptake of plasmid DNA can be enhanced by using a hand-held Swiss jet injector, which uses pressurized air to force small volumes (3-10μl) of naked DNA into targeted tissues [Walther et al., 2004]. The process by which plasmids are introduced into E coli is known as transformation. Transformation is a very important tool in recombinant DNA technology. The addition of new genes to a recipient cell introduces a heritable modification in the recipient cell's phenotype.

1.1.3 Gene therapy approaches

The gene therapy approaches currently being employed can be divided into four major categories [Zwacka and Dunlop, 1998] [Yamamoto et al., 2001]. They are (1) Enzyme/prodrug systems (suicide gene therapy) [Huang et al., 2005;Kerr et al., 1997] (2) Gene correction (tumour suppressor gene replacement therapy and oncogene inactivation) [Meng and El Deiry, 1998] (3) Immune-gene therapy and (4) Drug resistance gene therapy.

1.1.4 Basis of gene therapy in colorectal cancer

Virus-directed enzyme-prodrug therapy (VDEPT)

VDEPT is the gene transfer of bacterial or viral enzymes into tumour cells which can convert inactive prodrug into short lived toxic metabolites, limiting the toxic effects to the tumour cells [Walther et al., 2005]. Examples include: (1) Thymidine kinase and ganciclovir; (2) Cytosine deaminase and 5-fluorocytosine; and (3) Nitroreductase and the prodrug CB1954 [Chung-Faye et al., 2001]. One of the major mechanisms of the efficacy of this form of therapy is the so called 'bystander effect' [Chung-Faye et al., 2000]. This is a phenomenon by which small molecules, such as

an active drug metabolite, are able to pass between cells via gap junctions so that untransfected cells are also affected. Thymidine kinase of herpes simplex can phosphorylate ganciclovir, which can inhibit DNA polymerase leading to cell death. An animal experiment on colorectal cancer showed very profound bystander effects when the combination of thymidine kinase/ganciclovir was used [Link, Jr. et al., 1997].

Gene correction

Cancer may result when there is an imbalance between proto-oncogenes and tumour suppressor genes. Gene corrective therapy is aimed at reversing some of the genetic abnormalities by either introducing a tumour suppressor gene or inactivating proto-oncogene by an anti-sense method [Rachel Hargest, 2000]. Tumour suppressor gene TP53 is the most commonly mutated gene in human cancer [Linderholm et al., 2006] which is present in 20-69% of colorectal cancers. Its product known as 'p53' normally regulates the cell cycle and repairs abnormal DNAs. If the DNA cannot be repaired by the p53, it causes growth arrest or apoptosis [Liu and Gelmann, 2002]. Loss of p53 leads to uncontrolled and aggressive cellular growth. In a phase I controlled trial, an adenovirus encoding wild type TP53 gene was delivered by hepatic artery infusion to 16 patients with TP53 mutated colorectal liver metastases [Chung-Faye and Kerr, 2000]. The side effects were fever and transient derangement in liver function. Although the gene was expressed in subsequently resected tumours, there was no significant change in radiographic appearance of the tumours. Another gene known as K-ras is also often mutated in colorectal cancer [Noda et al., 2006]. It encodes for a protein called p21 which is involved in cell signal induction and the control of cell proliferation. The K-ras mutations can be detectable in DNA purified from the stool [Sidransky et al., 1992]. Anti-sense oligonucleotides are specific

sequences which bind to complementary mRNA and prevent its translocation. Anti - sense Bcl-2 has been shown to potentiate apoptosis in lymphoma [Keith et al., 1995] and anti-sense K-ras is used in trials on patients with lung cancer.

Immune-gene therapy

Immunological mechanisms are important for elimination of cancer by human body. Individuals with immune deficiencies, such as HIV infection, are at high risk of developing cancers. Cancer cells are recognized and destroyed by CD 8+ cytotoxic T cells and natural killer cells. But many cancer cells escape this immune mediated destruction by exhibiting loss of HLA class I antigens. A Phase 1 trial of immunotherapy for colorectal metastases using intra-lesional injection of HLA-B7 cDNA with liposome on 15 patients did not show any therapeutic benefit [Rubin et al., 1997]. Carcinoembryonic antigen (CEA) is often expressed by colorectal cancers. Immunizations with dendritic cells (DC) transfected with RNA encoding tumour antigens, induce potent tumour antigen-specific immune responses *in vitro* and in murine models. In a Phase II study [Morse et al., 2003] on patients with resected hepatic metastases of colon cancer, the safety and feasibility of administering autologous DC loaded with CEA mRNA was assessed. The immunizations were well tolerated. 9 out of 13 patients relapsed at a median of 122 days. Evidence of an immunologic response was demonstrated in biopsies of DC injection sites and peripheral blood of selected patients.

Activation of cytotoxic lymphocytes and natural killer cells, using cytokines such as interleukin-2 and interleukin-12 which can be transferred directly into tumour cells could result in an anti-tumour effect [Caporale et al., 2007;Alves et al., 2004]. In a Phase 1/2 clinical trial on patients with unresectable colon cancers who were treated with intra-tumoral injection of an adenovirus-IL-2 at the time of surgery, showed that

one patient's tumour expressed increased numbers of membrane bound IL-2 receptors. Another patient showed necrosis of the tumour mass. In a study on mice with experimentally induced colorectal tumours, adenovirus containing mouse IL-12 gene was injected into the tumour and it showed tumour regression and prolonged survival [Caruso et al., 1996].

Drug resistance gene therapy

The main limiting factor for patients undergoing chemotherapy is bone marrow toxicity. There is a gene called multiple drug resistance gene (MDR1) which may confer the bone marrow resistance to vinca alkaloids, anthrocyclins and paclitaxel [Ueda et al., 1986]. This gene therapy is still in an experimental stage.

New gene therapy to inhibit growth factors

The method described here is a different approach to the above, as it involves gene transfer of a binding protein that inhibits the effect of IGF-I. This gene therapy was evaluated in this project.

1.1.5 Current colorectal gene therapy trials

There are several gene therapy trials happening in the world. The trials which are underway in the UK are shown below *.

1) A pilot study of recombinant CEA vaccinia virus vaccine with post vaccination CEA peptide challenge in combination with 5-fluorouracil and folinic acid in the treatment of colorectal cancer (Phase I subcutaneous).

2) Evaluation of the safety, biodistribution and efficacy of TroVax in patients with metastatic colorectal cancer (Phase I i.m) (Vaccinia virus and oncofetal antigen).

3) Phase II study of TroVax in colorectal cancer patients undergoing surgery for resectable liver metastases (Pox virus and oncofoetal antigen).

4) Gene therapy for bowel cancer that has spread to the skin (Granulocyte – macrophage colony stimulating factor gene and herpes simplex virus).

5) Gene therapy protocol for the evaluation of the safety and efficacy of TroVax in conjunction with chemotherapy in patients with metastatic colorectal cancer.

6) Phase I/II study of DNA vaccination with a CEA/pDOM fusion gene in patients with carcinoma expressing CEA (CAP-1 peptide from CEA and naked plasmid DNA).

7) A Phase II study immunologically evaluating 5T4-MVA (TroVax) in patients undergoing surgical resection of colorectal liver metastases (Vaccinia and oncofoetal antigen).

8) A randomised Phase II trial assessing anti-CEA, anti-MUC-1 vaccination +/- chemotherapy +/- GM-CSF after surgery in patients with stage II colorectal cancer (Poxvirus, Vaccinia virus and Carcino embryonic antigen /MUC-1).

9) Safety and Immunology evaluation of TroVax produced by the Baxter synthetic route in patients with stage IV colorectal carcinoma.

10) A Phase I study of adoptive transfer of autologous tumour antigen-specific T cells with pre-conditioning chemotherapy and intravenous IL-2 in patients with advanced CEA positive tumours (retrovirus and CEA plus CD3).

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Summary

Gene therapy trials are ongoing and the results are eagerly awaited. My current experiment attempts to alter the components of the IGF system in a colon cancer model by gene transfer. Therefore, the IGF system in colorectal cancer will be discussed in the next chapter.

1.2 IGF system and its role in colorectal cancer

The purpose of this literature review is to provide an overview of the IGF system.

1.2.1 Biology of the IGF System

The IGF system includes ligands IGF- I and - II, their receptors, IGF-binding proteins (IGFBPs) and IGF proteases. Figure 1.1 is a schematic representation of the IGF system. Their biological features and actions are summarised in Table 1.1.

IGF-I and IGF-II

The IGFs are single-chain polypeptides with structural homology to pro-insulin. The IGF-I is a basic peptide with 70 amino acids and the IGF-II is slightly acidic with 67 amino acids. IGF-I and IGF-II act on a variety of mammalian cells in an endocrine, paracrine and autocrine manner [Grimberg and Cohen, 2000;Hallberg et al., 2000] to regulate cell proliferation, apoptosis, transformation and differentiation [Leng et al., 2001b;Moschos and Mantzoros, 2002]. Both IGF-I and -II are essential for normal human growth and development [Zhou et al., 2003]. Growth hormone regulates the expression of IGF-I gene and it thereby mediates most of its actions through IGF-I, but it does not control the expression of IGF-II [Yu and Rohan, 2000]. Adults have a higher concentration of IGF-II than IGF-I, but it is the IGF-I that plays an important role in postnatal growth, while the IGF-II acts mainly in embryonic and foetal life [Yu and Rohan, 2000]. The majority of circulating IGFs, in particular IGF-I, is produced by the liver, although various tissues have the capability to synthesize these peptides locally [Khandwala et al., 2000;Moschos and Mantzoros, 2002]. More than

90% of the IGFs are bound to IGFBP-3 [Giovannucci, 2001;Grimberg and Cohen, 2000] and only 1% of the IGF circulates in the free form.

IGF receptors

The actions of IGFs are mediated via type I and type II receptors (IGF-IR and IGF-IIR), both of them glycoproteins, found in the cell membrane. The growth-promoting effects of IGFs are mediated through the IGF-IR, which contains a tyrosine-kinase domain resembling insulin receptor[Grimberg and Cohen, 2000]. The IGF-IR has two extracellular subunits (α) to bind the IGFs, and two intracellular subunits (β) which contain tyrosine-kinase [Reinmuth et al., 2002b] to mediate the intracellular effects. In contrast to the insulin receptor, which primarily mediates metabolic function, the IGF-IR mediates mainly growth and differentiation [Blakesley et al., 1996]. When activated the IGF-IR stimulates the synthesis of RNA and DNA, cell proliferation, differentiation and increases cell survival [Reinmuth et al., 2002b]. The IGF-IIR is similar to mannose-6 phosphate receptor. It is a single chain polypeptide with a large extracellular domain, a single transmembrane region and a small cytoplasmic domain. It is entirely different from the IGF-IR in that it has no tyrosine-kinase activity. The IGF-IIR is thought to function as a clearance receptor for IGF-II, thereby influencing the extracellular levels of the IGF-II [LeRoith and Roberts, 2003]. The IGFs can also act via a hybrid receptor, which consists of a single α and β subunit linked by disulfide bonds. These hybrid receptors have half insulin hemi receptor and a dimerized IGF-IR. The IGF-IR/hybrid receptors retain the high affinity for IGF-I, but exhibit a dramatically decreased affinity for insulin [LeRoith and Roberts, 2003].

IGF-binding proteins (IGFBPs) and IGF proteases

The effects of the IGFs are modulated by at least 10 different IGF binding proteins [Kim et al., 1997;Kostecka and Blahovec, 1999], six of which have a high affinity for IGFs (IGFBP1-6) and four of them have a low affinity for IGF [Baxter et al., 1998;Kim et al., 1997], also known as IGFBP-related proteins (IGFBP-rp1-4). Table 1.2 summarises the facts related to IGFBPs 1-7.

The IGFBPs are a family of homologous proteins that are produced by many different tissues and all have different molecular weight, amino acid composition, binding properties and distribution in biological fluids [Kostecka and Blahovec, 1999], but they all share cysteine-rich N terminal domain and C-terminal [Kim et al., 1997]. They also have IGF-independent actions [Kim et al., 1997]. The IGFBPs control the distribution of IGFs because of their higher affinity to IGFs, which is 2-50 times higher than that of the IGF-IR [Mazerbourg et al., 2004]. IGFBP-1-5 preferentially bind the IGF-I over the IGF-II, but IGFBP-6 has 100-fold higher affinities for IGF-II than for IGF-I[Grimberg and Cohen, 2000]. The IGFBPs do not bind to the insulin [Kostecka and Blahovec, 1999;LeRoith and Roberts, 2003]. The key function of the IGFBPs is regulation of circulating bioavailability of the IGFs [Baxter, 2000]. The IGFBPs serve as carriers, mediators as well as reservoirs of IGFs by protecting IGFs from degradation and delivering them to appropriate tissue [Baxter et al., 1998;Henric Zazzi, 1998;Zazzi et al., 1998]. IGFBP-3 is the main binding protein, which is found in circulation in abundance, and most of the IGFs are bound to it. The IGFBP-3 forms a ternary complex with IGF-I and a separate protein, namely 80 kDa acid-labile subunit [Pollak, 2000] to keep the IGF-I within the

vascular system [Sandhu et al., 2002], while the other IGFBPs form binary complexes with IGF-I, allowing transport of IGFs across the capillary to various tissues [Sandhu et al., 2002]. IGFBPs-1-6 inhibit the action of IGFs by high affinity binding while IGFBP-1, -3 and - 5 also potentiate the IGF effects [Baxter, 2000]. The potentiation needs a decrease in binding affinity of the IGFBP for IGFs so that the IGFs can act on the IGF-IR. This process involves association of IGFBP with the cell surface or extracellular matrix, and / or post-synthetic processing of IGFBP, e.g., phosphorylation and proteolysis [Remacle-Bonnet et al., 1997]. IGFBP-1, -3 and -5 have well-established effects that are independent of IGF-IR signalling. IGFBP-1 exerts these effects by signalling through [α] 5[β] 1-integrin, whereas IGFBP-3 and - 5 may have specific cell-surface receptors with serine kinase activity [Baxter, 2000].

The IGFBPs are degraded by several proteases, some of which are specific to that binding protein, and others are non-specific proteases that control the function and the tissue availability of IGFBPs. These proteases cleave the IGFBPs into fragments with lower affinity for the IGFs. In the serum proteolysis of the IGFBP-3, which is the main binding protein, is negligible in the normal population, but it is increased in certain physiological conditions like pregnancy and pathological conditions like the post-operative period and malignancies [Baciuchka et al., 1998], which increases the availability of circulating free IGFs. In tissues, IGFBP proteases enhance the IGF-I availability by cleaving IGFBP, thereby increasing free IGF-I concentration[Grimberg and Cohen, 2000]. Experiments show that proteolytic activity is highest in the proliferating cells [Kuemmerle and Teng, 2000]. The biological activity of IGF is determined by the integrated actions of circulating IGF-I and IGFBP, and by local production of IGF, IGFBP and IGFBP protease.

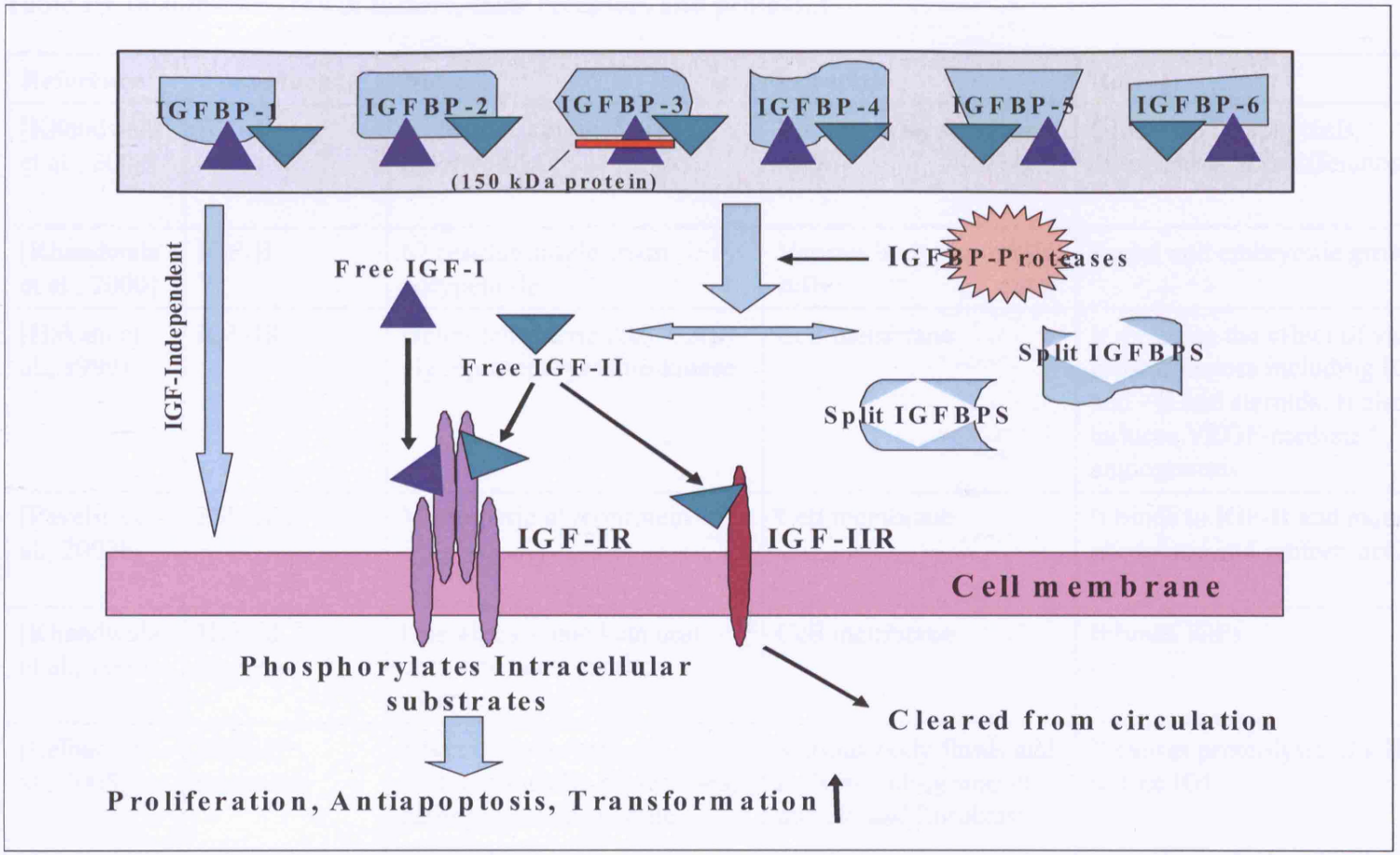


Figure 1.1 Schematic representation of insulin-like growth factor (IGF) system. The IGF system consists of two growth factors (IGF-I and -II), two receptors (IGF-IR & -IIR), 6 binding proteins and proteases. IGF-IR is proliferative in action while IGF-IIR clears the IGFs from circulation. IGFBP proteases destroy the binding proteins and increases local tissue availability of the IGFs.

Table 1.1 Insulin-like growth factors, their receptors and proteases

Reference	Components	Nature	Location	Role
[Khandwala et al., 2000]	IGF-I	70 residue single chain polypeptide	Various body fluids and cells	Growth, anti-apoptosis, mitogenesis and differentiation
[Khandwala et al., 2000]	IGF-II	67 residue single chain polypeptide	Various body fluids and cells	Foetal and embryonic growth
[Hakam et al., 1999]	IGF-IR	Heterotetrameric 2(α) + 2(β) glycoprotein-tyrosine-kinase	Cell membrane	It mediates the effect of various growth factors including IGF-I and - II and steroids. It also induces VEGF-mediated angiogenesis
[Pavelic et al., 2002b]	IGF-IIR	Monomeric glycoprotein	Cell membrane	It binds to IGF-II and mannose-6 phosphate and inhibits action of IGF-II
[Khandwala et al., 2000]	Hybrid receptor	One alpha + one beta unit with one hemi insulin receptor	Cell membrane	It binds IGFs
[Lelbach et al., 2005]	IGFBP* proteases	Several types (serine protease,metallo proteinases, cathepsins and specific proteinases)	Various body fluids and cells including smooth muscle and fibroblast	It causes proteolysis of IGFBP to free IGF

* IGFBP - insulin-like growth factor binding protein

Table 1.2 Insulin-like growth factor binding proteins

Reference	IGFBPs	Protein size (kDa)	Location [a]	Role [b]
[Baxter, 2000]	IGFBP-1	25 -34	Various body fluids including amniotic fluid	Modulate the IGF effects
[Clemmons, 1993]	IGFBP-2	32-34	Various body fluids including CSF	Mediates TNF-α induced apoptosis and stimulate cell proliferation
[Rajaram et al., 1997]	IGFBP-3	53	Various body fluids	Main carrier protein for IGFs in circulation & growth inhibitor. 90% of serum IGF is bound to IGFBP-3
[Zhou et al., 2003]	IGFBP-4	24, 29	Various body fluids and tissues including liver, vascular smooth muscle	Inhibits the actions of IGFs
[Rajaram et al., 1997]	IGFBP-5	23	Various body fluids, osteoblasts, kidney, etc.	Increases mitogenesis
[Baxter, 2000]	IGFBP-6	30 -32	Serum and CSF	Modulates the actions of IGFs
[Degeorges et al., 2000]	IGFBP-7	27	Various tissues including smooth muscle and endothelium	Modulates the actions of IGFs

All IGFBPs can act as a carrier for IGFs. [a]All IGFBPs are found in serum

[b]All IGFBPs except IGFBP-4 and 6 have both IGF potentiating as well as inhibiting actions under different circumstances.

1.2.2 Studies linking the IGF system and colon cancer

Tables summarising the results of population-based, *in vitro* and *in vivo* studies linking the IGF system and colon cancer are shown in the appendix. Most of the current knowledge about the IGF system is based on *in vitro* and population-based studies. Most population-based studies measured a few factors in the serum, and compared them with the incidence or development of colorectal adenoma and carcinoma. Some of the studies are larger and others are smaller. Not all of them clearly support a linear association between IGF-I, IGF-II, IGFBPs and colorectal cancer. It may be due to the fact that the relation between these factors may be a complex one. Some studies show no association between plasma IGFBP-1 and IGFBP-2 and risk of colon and/or rectal cancer [Palmqvist et al., 2003], while others show that chronically high levels of circulating insulin and IGFs associated with a Western lifestyle may increase the colorectal cancer risk, possibly by decreasing the IGFBP-1 and increasing the bioactivity of the IGF-I [Kaaks et al., 2000]. Overall, the studies suggest that an increase in the IGF-I and a decrease in the IGFBP-3, leading to increased serum free IGF-I, may be associated with the development of colorectal adenoma and carcinoma [Nomura et al., 2003;Teramukai et al., 2002]. Renehan [Renehan et al., 2004] performed a meta-regression analysis of case-control studies linking the relationship between IGF-I, IGFBP-3 and cancers. Their analysis showed that a higher concentration of IGF-I was associated with colorectal cancer, but a high concentration of IGFBP-3 did not protect from colorectal cancer. It is possible that the case-control studies might have overestimated the relationship between IGFs, IGFBP-3 and colorectal cancer.

The levels of circulating IGFs and IGFBPs have been shown as varying significantly between studies. The method of collection (serum or EDTA) has been suggested to be a source of heterogeneity [Renehan et al., 2003]. It has been found that the levels of IGFs were about 10% higher in serum than in plasma, the differences were statistically significant ($P<0.001$). IGF levels correlated well between serum and plasma samples ($r \geq 0.95$, $P<0.001$). The IGFBP-3 levels were not different between serum and heparin plasma ($P=0.75$), but were significantly higher in these two specimens than in EDTA plasma ($P<0.001$). IGFBP-2 levels were significantly different among the three types of specimen ($P<0.001$). The values were 83% higher in EDTA specimen than in heparin plasma. IGFBP-6 levels were significantly higher in serum than in plasma ($P<0.01$). These indicate the fact that the method of collection can influence the results of the study related to the IGF system. So there is a need to standardize the method of collection, which will enable us to compare different studies and their significance. The results of *in vitro* and *in vivo* studies linking the IGF system and colon cancer are summarised under different headings down below.

1.2.3 Pathophysiological role of the IGFs

The IGF system plays a critical role in all phases of mammalian growth including intrauterine, childhood and puberty [Rosenfeld, 2003]. This is confirmed by the evidence that targeted disruption of mouse gene for IGF-II resulted in reduction of foetal growth, but normal postnatal growth and disrupted IGF-I gene lead to a similar decrease in birth weight but also persistent postnatal growth failure [Rosenfeld, 2003]. Liver is the most frequent site of metastasis after colon cancer [Reinmuth et al., 2002a], which may be due to the fact that it is the liver that produces most of the

IGFs in circulation. The IGF-I has profound impact on tumour growth by stimulation of cellular proliferation and inhibition of apoptosis. Wu et al. conducted a study involving liver-specific IGF-I-deficient (LID) mice, in which serum the IGF-I level was 25% of that in control mice. In LID mice, the growth of orthotopically transplanted colon adenocarcinoma was significantly less when compared with controls (31.3 versus 56.8%, $P<0.01$), and the appearance of a palpable caecal tumour not only slower ($P<0.05$) but also smaller ($P<0.01$) than that in control mice. The frequency of hepatic metastasis was also significantly lower in LID mice (31.3% versus 44.0% in control mice, $P<0.05$). These results support the hypothesis that circulating IGF-I levels play an important role in tumour development and metastasis [Wu et al., 2002]. Increased systemic IGF-I may lead to colonic tumours as evidenced in acromegaly patients. Malnutrition and calorie restriction may lower the level of the IGF-I which may delay tumour progress. The IGF-I also plays an important role in tissue angiogenesis [Akagi et al., 1998]. It upregulates VEGF expression in human colon cancer cells, which is mediated through hypoxia-inducible factor-1, [Fukuda et al., 2002] without involving IGFBP [Collard et al., 2003]. Colorectal cancers often express 10-50 times higher levels of IGF-I and IGF-II than adjacent uninvolved colonic mucosa [Reinmuth et al., 2002a] although some studies found normal IGF-I levels [Freier et al., 1999]. IGF-I has a biphasic effect on colon cancer cells initially mitogenic, then mediating growth arrest and differentiation [Oh et al., 2001]. The IGF-I may increase the risk of cancer either by its anti-apoptotic activity or by modulating the effects of sex steroids [Smith et al., 2000].

The IGF-II also has a place in the development of colon cancer. Experiments conducted by Zarrilli showed high levels of IGF-II mRNA in proliferating Caco-2

cells, which decreased by more than 10-fold when the cells ceased to proliferate and differentiate [Zarrilli et al., 1994]. Reduced IGF-II expression was associated with a decrease in IGF-I receptor numbers that were high in proliferating cells. Exogenously added IGF-II was able to stimulate proliferation of serum-deprived cells in a dose-dependent fashion [Zarrilli et al., 1994]. Increased systemic IGF-II level does not have any predictive influence on susceptibility of colorectal cancer [Hassan and Macaulay, 2002]. The IGF-II acts on the IGF-I receptor in an autocrine/paracrine manner, and monoclonal antibody to IGF-IR(α-IR3) inhibits both basal and IGF-II-stimulated cell proliferation [Manousos et al., 1999].

The IGF-IR is crucial for normal growth and development because IGF-IR knockout mouse embryo suffer generalised organ hypoplasia and invariably dies at birth [Reinmuth et al., 2002b]. The IGF receptors are found in both mucosal and muscular layers of intestine and are mainly concentrated in the basolateral region of crypt enterocytes [Howarth, 2003]. Experimental administration of IGF-I in rats showed an increase in crypt cell population, as well as linear and cross sectional increases in mucosal and muscular layers of intestine, leading to increase in gut weight [Howarth, 2003]. The IGF-IR is essential for cell transformation that is induced by tumour-virus proteins and oncogene products [Yu and Rohan, 2000]. Colorectal cancer cells often over express IGF-I receptors [Ouban et al., 2003] and when activated by IGF-I, they inhibit apoptosis and allow progression through the cell cycle. Thus, IGF-I can influence both pre-malignant and cancerous stages [Giovannucci, 2001]. Cancer cells with a strong tendency to metastasize have a higher expression of IGF-IR [Hakam et al., 1999;Yu and Rohan, 2000]. Hakam [Hakam et al., 1999] investigated the expression of IGF-IR in colonic adenomas, adenocarcinomas and in

corresponding metastases. Immunostaining for IGF-IR showed strong cytoplasmic positivity in 96% (34/36) carcinomas, and in 93% (25/27) metastases. Only a faint cytoplasmic stain of the IGF-IR was identified in 83% (10/12) adenomas while normal mucosa showed negative immunostaining. But another study did not find any difference in the IGF-IR concentration when they compared 46 frozen sections of colon cancer with 26 controls [Adenis et al., 1995]. IGF-IR activation up-regulates the components of the TGF-alpha autocrine loop resulting in TGFα-mediated EGFr activation, which was critical for the IGF-IR mediated re-entry into the cell cycle from the growth-arrested state [Wang et al., 2002].

The IGF-IIR antagonises the growth promoting effect of IGF-II, and loss of IGF-IIR is expected in cancer [Yu and Rohan, 2000]. The IGF-IIR acts like a clearance receptor and removes the IGF-II from the circulation. Experiments show that there is a rise in the IGF-II/Man-6-P receptor message in colorectal cancer, and the increase in IGF-II message is accompanied by a doubling of the IGF-II protein in the tumour tissue, compared with the adjacent normal tissue [Freier et al., 1999]. Garrouste isolated soluble IGF-II/mannose 6-phosphate receptors in the culture medium from HT-29 colonic cancer cells [Garrouste et al., 1991]. These findings suggest that the IGF-II/Man-6-P receptor may also be involved in the development of adenocarcinoma of the colon.

The IGF-I, IGF-II and IGF-IR are often over-expressed by many cancers, so that IGF-I and IGF-II can act in an autocrine manner [Guo et al., 1995;LeRoith and Roberts, 2003;Weber et al., 1999]. Most colonocytes secrete IGF-II as well as express the IGF-IR, but it is the sequestration of IGF-II by the IGFBPs that prevents

the establishment of an IGF autocrine loop. One study examined the IGF-IR expression by the colon of Fischer 344 rats. Colonic IGF-IR mRNA levels declined with ageing ($P \leq 0.05$) while colonic IGF-I mRNA levels were unchanged, which may be a protective adaptive mechanism against ageing colon [Hallberg et al., 2000]. Insulin, IGF-I and II induce expression of hypoxia-inducible factor-1, which is required for expression of genes encoding IGF-II, IGFBP-2 and IGFBP-3 [Feldser et al., 1999]. Colon cancer cells produce specific proteases that degrade the IGFBP secreted by these cells [Michell et al., 1997b] so that there will be free IGFs for completing autocrine loop.

1.2.4 Genetic changes and colon cancer carcinogenesis

Numerous mutagenic events can occur throughout the colon cancer development including loss of heterozygosity in tumour suppressor genes such as APC, MCC, DCC, p53 and K-ras [Takami et al., 1995]. Colon cancer tumorigenesis is a step-wise process [Cho and Vogelstein, 1992] in which mutations accumulate over time and oncogenes are activated while tumour suppressor genes are deactivated. Colon cancer mechanisms are discussed as common and alternate pathways.

Common pathway

Tumour suppressor genes make proteins that suppress tumour formation by limiting the cell growth. Mutations involving the tumour suppressor genes could result in a loss of their ability to restrict tumour growth [Samowitz et al., 2007;Allen, 1995]. Vogelstein and his colleagues found that mutations involving a tumour suppressing gene called the adenomatous polyposis coli (APC) gene resulted in colon cancer [Baba, 1997]. Normally, APC binds to b-catenin and phosphorylates it. But when

mutations occur, b-catenin interacts with transcription factors instead of stimulating growth. P53 is another tumour suppressor gene which blocks the cell cycle and stimulates apoptosis. It is altered in more than 80% of colorectal cancers.

Alternate pathway

Hereditary non polyposis coli (HNPCC), sometimes called Lynch syndrome, accounts for approximately 5 - 10% of all colorectal cancer cases. Several genes have been identified that are linked to HNPCC. Mutations in the *MLH1*, *MSH2*, and *MSH6* genes are the most frequent cause of HNPCC [Rahner et al., 2007;Plotz et al., 2006]. Although multiple genes have been linked to HNPCC, most families with HNPCC have a mutation in only one of the genes. Genetic testing is available for the *MLH1*, *MSH2*, and *MSH6* genes. The HNPCC genes are part of a group of genes called mismatch repair genes. They make proteins that repair DNA mistakes that occur as cells divide. If one of these genes has a mutation, the mistakes cannot be repaired, leading to damaged DNA and an increased risk of cancer. The risk of colorectal cancer in families with HNPCC is 70 - 90%, which is several times the risk in the general population. Women with HNPCC also have an increased risk of cancers of the uterus, ovaries, stomach, small intestine, kidney and breast [Kwak and Chung, 2007].

1.2.5 Components of the IGF axis and colon cancer tumorigenesis

Acromegaly is a condition of growth hormone excess and is associated with increased proliferation of normal colonic epithelium [Palmqvist et al., 2003], and increased prevalence of tubulovillous adenoma and colon carcinoma [Miraki-Moud et al., 2001]. Growth hormone increases serum IGF-I levels [Cohen et al., 2000]. In

a retrospective study [Miraki-Moud et al., 2001], serum IGF-I, IGF-II, IGFBP-2 and IGFBP-3 in patients with acromegaly, and in those with colonic neoplasia without acromegaly, were analysed. Mean serum IGF-I and IGFBP-3 levels were significantly elevated in patients with acromegaly without colonic neoplasia, and in those with acromegaly and colonic neoplasia, and significantly reduced in those with colonic neoplasia without endocrine disease, compared with controls ($P<0.001$). However, median serum IGFBP-2 levels were significantly elevated in patients with acromegaly and colonic neoplasia ($P<0.01$), and in those with colonic neoplasia without endocrine disease ($P<0.0001$). The precise mechanism of development of colon cancer in acromegaly remains unknown. It may be due to the stimulatory role of excess growth hormone /IGF-I or excess IGF-II [Miraki-Moud et al., 2001]. In one study involving acromegaly, 5% of patients had colorectal cancer and 25% had colorectal adenoma [Jenkins et al., 2000]. Antagonists to growth hormone releasing hormone (GH-RH) inhibit the growth of HT-29 human colon cancers both *in vitro* and *in vivo* [Szepeshazi et al., 2000]. The effect of GH-RH antagonists may be mediated through reduced production and secretion of IGF-II by cancer cells. A GH transgenic mouse does not develop cancer, even though the serum IGF-I level is high.

All human genes have two copies, one is paternal and the other is maternal in origin. Among these two copies of gene, only one is functional and this phenomenon is termed genomic imprinting. Imprinting is vital for normal development, and disruption of imprinting mechanisms gives very similar phenotypes in mice and humans [Brenton et al., 1995]. The IGF-II gene is imprinted and expressed only from paternal allele, except from the adult liver, where the expression is biallelic because of promoter switching after birth [Hu et al., 1996]. The IGF-IIR is also imprinted but

expressed only from the maternal allele. This may mean maternally-expressed genes can act as growth suppressors because the function of IGF-IIR is to remove the IGF-II from circulation and destroy it so that less IGF-II is available to promote cell growth [Haig and Graham, 1991;Jirtle, 2004]. Loss of genomic imprinting (LOI) of the IGF-II gene involves an abnormal activation of the normally silent maternally-inherited allele and as a result the cells produce double the dose of IGF-II [Cruz-Correa et al., 2004]. LOI of the IGF-II gene is found in normal colonic mucosa of about 30% of colorectal cancer patients, but it is found in only 10% of healthy individuals.

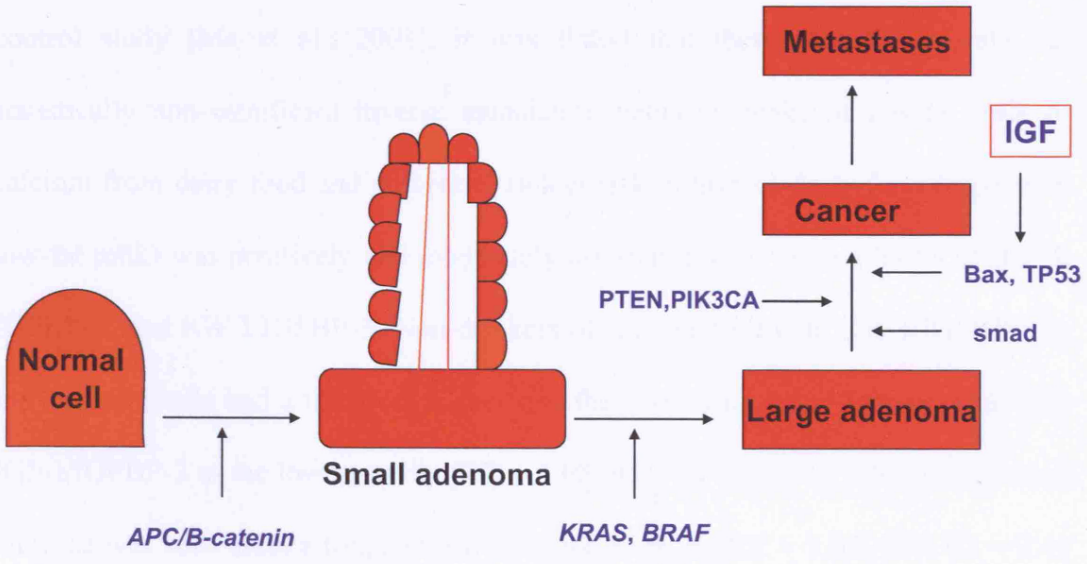


Figure 1.2 Vogelgram showing molecular mechanisms involved in the pathogenesis of colorectal cancer. Small adenoma increases in size under the influence of growth controlling pathways. Large adenoma becomes cancer under the influence of various genes and growth factors including IGF.

Colon cancer development is a multistep process (fig 1.2). Vogelstein proposed a mechanism of development of hereditary colon cancer. It involves a series of mutations. Mutation of APC causes small adenoma formation. Subsequently it becomes large adenoma when K-ras mutation occurs. Large adenoma becomes cancer when there is mutation of genes such as P53. The exact role IGF in cancer formation is unclear. It appears that IGFs influence the cancer formation by acting as an anti-apoptotic signal which may be mediated by Bax proteins.

1.2.6 Factors influencing the IGF system

Dairy products

Intake of dairy products is associated with an increase in circulating IGF-I levels, but intake of low fat milk is associated with a lower risk of colorectal cancer, particularly among individuals with high IGF-II/IGFBP-3 [Ma et al., 2001]. In a nested case-control study [Ma et al., 2001], it was found that there was a moderate but statistically non-significant inverse association between intake of low-fat milk or calcium from dairy food and colorectal cancer risk. Intake of dairy food (especially low-fat milk) was positively and moderately associated with plasma levels of IGF-I, IGFBP-3, and IGF-I/IGFBP-3. Non-drinkers of low-fat milk with IGF-I/IGFBP-3 in the highest tertile had a threefold higher risk than non-drinkers of low-fat milk with IGF-I/IGFBP-3 in the lowest tertile (RR = 3.05; 95% CI = 1.29 to 7.24), but no such increase was seen among frequent low-fat milk drinkers (RR = 1.05; 95% CI = 0.41 to 2.69). There was a statistically significant interaction between low-fat milk intake and IGF-I/IGFBP-3 in association with risk of colorectal cancer (P =.03). Men with high IGF-I/IGFBP-3 who were frequent low-fat milk drinkers had a 60% lower risk (95% CI = 0.17 to 0.87; P =.02) than non-drinkers of low-fat milk.

Chronic energy restriction reduces plasma levels of IGF-I, and IGFBP-3, increases plasma IGFBP-1 and IGFBP-2, and thereby protects from various cancers [Palmqvist et al., 2003]. On the other hand, obesity causes hyperinsulinaemia and reduction in plasma IGFBP-1 and IGFBP-2 [Palmqvist et al., 2003]. In one study, the mean serum IGF-I concentration was 13% lower in 92 vegan women, compared with 99 meat-eaters and 101 vegetarians ($P = 0.0006$) [Allen et al., 2002]. The mean concentrations of both serum IGFBP-1 and IGFBP-2 were 20-40% higher in vegan women compared with meat-eaters and vegetarians ($P = 0.005$ and $P = 0.0008$ for IGFBP-1 and IGFBP-2, respectively). Cohort studies show that dairy products and milk protect against colon cancer, but the evidence has not been supported by case-control studies [Norat and Riboli, 2003]. No proven evidence is available regarding whether cheese and yoghurt protect against colon cancer [Norat and Riboli, 2003] through regulation of the IGF system.

Non-steroidal anti-inflammatory drugs

Non-steroidal anti-inflammatory drugs (NSAIDs), which may inhibit the activity of cyclooxygenase-2 (COX-2), reduce the IGF-IR expression in colon cancer lines, and inhibit IGF-II-stimulated growth and invasion in a dose-dependent manner [Yasumaru et al., 2003]. These changes are reversible when treated with prostaglandin E2 (PGE_2) or angiotensin II [Yasumaru et al., 2003]. Therefore, combination therapy with NSAIDs and ACE inhibitors targeting IGF-IR might be a novel and potentially promising strategy for the chemo-prevention of colon cancer. There is also a direct correlation between COX-2 and IGF-II expression in Caco-2 cells. The IGF-II up-regulates COX-2 expression and PGE_2 synthesis in Caco-2 cells, which is mediated by IGF-IR [Di Popolo et al., 2000]. This is supported by the

evidence that when IGF-IR is blocked with an antibody (α-IR3), it results in not only inhibition of COX-2 expression and PGE_2 synthesis, but also induction of apoptosis in Caco-2 cells [Di Popolo et al., 2000].

Other chemicals

Retinoic acid and dexamethasone cause growth inhibition of colon cancer cells *in vitro,* which is accompanied by increased IGFBP-2 expression and specific IGFBP-2 proteolysis, resulting in reduced affinity for IGF-II [Hoflich et al., 1998]. Suramin releases IGF-II from the IGF-II-IGFBP complex so that IGF-II can act on the IGF-IR of colon cell line [Pommier et al., 1992]. Intestinal epithelial cells respond to short-chain fatty acids by altering secretion of IGFBPs [Nishimura et al., 1998]. Short-chain fatty acids are bacterial metabolites from unabsorbed carbohydrate. Trans retinoic acid (Tra) inhibits Caco-2 cell proliferation in a dose-dependent manner via increased expression of IGFBP-6, whereas it decreases the concentrations of IGFBP-2 and IGFBP-4. The IGF-IR expression on colorectal adenocarcinoma cell lines is decreased by chemopreventive agent N-acetyl-l-cysteine [Kelly et al., 2002]. Conjugated linoleic acid (CLA) inhibits cell proliferation and induces apoptosis in HT-29 cells [Kim et al., 2003]. Levels of IGFBP-3, which may induce apoptosis by IGF-dependent or –independent mechanisms, are increased by butyrate and trichostatin A and the IGF-II augments this effect [Leng et al., 2001a]. Butyrate also increases the secretion of IGFBP-2 in a dose-dependent and reversible manner [Leng et al., 2001a;Nishimura et al., 1998].

Chemo-radiation

Cell culture studies show that IGF-I receptor activation blocks the expected cytotoxic effect of 5 FU and external beam irradiation [Perer et al., 2000]. IGF-IR antagonism using monoclonal antibody combined with chemo-radiation, produces an increased cytotoxic response [Perer et al., 2000], which may be useful in humans in the near future.

Diabetes

Patients with type 2 diabetes mellitus are at high risk of developing colorectal cancer [Meyerhardt et al., 2003]. Elevated levels of post-prandial insulin and C-peptide increase the colorectal cancer risk [Meyerhardt et al., 2003]. Insulin may directly activate its own receptor, the receptors for IGF-I, hybrid insulin/IGF-I receptor, or it may act indirectly by increasing the bioavailability of IGF-I by altering the IGFBP levels [Sandhu et al., 2002].

1.2.7 IGF related properties of human colon cancer cells

Heterogeneity

Different cancer cells behave in a different way with respect to IGF responsiveness. Virtually all human colon cancers express IGF-I receptors, but only 50% responds to growth and the mitogenic effects of exogenous IGF-I [Guo et al., 1992;Singh et al., 1994b]. Therefore, cancer cell lines can be divided into IGF-responsive (e.g. COLO 205, Caco-2) and IGF-non responsive (e.g. DLD-1) groups [Singh et al., 1994b]. Some colon cancer cells (e.g. LIM 1215) are IGF-responsive, but the IGF-II is not a major autocrine factor for these cells. All these suggest the existence of heterogeneity

between colon carcinoma cell lines with respect to the role of the IGF system [Leng et al., 2001b].

Polarity

Colon cancer cells behave in a polarised fashion and secrete different members of the IGFBPs from different areas of the cell surface. Caco-2 cells secrete IGFBP-3 from the apical surface, IGFBP-2 predominantly from the basolateral surface, and IGFBP-1 and -4 from both surfaces. However, epidermal growth factor (EGF) induces secretion of the IGFBP-4 more from the apical surface than from the basolateral aspect. It does not affect the polarity of the other IGFBPs [Oguchi et al., 1993]. HT29-D4 human colonic carcinoma cells secrete endogenous IGF-II predominantly (66%) from the basolateral cell surface, where type I IGF receptors are almost all (>96%) localized. IGFBP-2 and IGFBP-4 are secreted primarily into the basolateral side (71 and 87%, respectively); whereas IGFBP-6 is targeted to the apical surface (76%). The differential sorting of the various forms of IGFBPs may play a modulatory role in the maintenance of functional polarity in the differentiated colon cancer cells [Remacle-Bonnet et al., 1992].

Differential expression

The majority of human colon cancer cells express IGF-II, IGFBP-2 and IGFBP-4 and less frequently IGFBP-3, IGFBP-5 and IGFBP-6; the levels of IGFBP-2 and IGFBP-6 decrease as differentiation proceeds (differentiation-dependent manner)[Mishra et al., 1998;Moschos and Mantzoros, 2002;Singh et al., 1996;Zhang et al., 1995]. Singh et al. measured the expression and secretion of IGF-II, IGFBP-2, and IGFBP-4 in relation to growth and differentiation of Caco-2 human colon cancer cells, which

undergo spontaneous enterocytic differentiation in culture. Caco-2 cells demonstrated an initial rapid phase of growth followed by a significant retardation in the growth. Changes in growth and differentiation were accompanied by a > 80% decline in the relative concentration of IGF-II mRNA. In contrast, the relative mRNA concentrations of inhibitory binding proteins (IGFBP-2 and IGFBP-4) increased rapidly to 200% of day 2 values by days 5-7, before returning to baseline levels by day 13 [Singh et al., 1996]. The quantity and the type (differential expression) of IGFBPs and IGF-II may play a critical role in both proliferation and differentiation of colonocytes [Singh et al., 1994b;Singh et al., 1996]. HT29-D4 cancer cells remain in an undifferentiated state in culture medium when they cannot use endogenous IGF-II as an autocrine regulatory factor [Remacle-Bonnet et al., 1992]. The IGF-IR primarily controls the differentiation of colonic cells [Oh et al., 1996].

1.2.8 Individual IGFBPs and their role in colorectal cancer

Among the IGFBPs, IGFBP-4 is the only inhibitory binding protein which is consistently inhibitory to cell growth. The IGFBP-4 is secreted by almost all colon cancer cell lines [Dai et al., 1997]. This protein has 40-50 times higher affinity to IGFs than IGF-IR. No previous studies available to date to assess its inhibitory nature on colon cancer *in vivo*. Therefore it will be discussed in detail in a separate section. The remaining IGFBPs are discussed down below.

IGFBP-1

IGFBP-1 is mainly produced by the liver, which is regulated by insulin [Sandhu et al., 2002]. It has equal affinity for IGF-I and IGF-II. It can either potentiate or inhibit the action of the IGFs [Hunt et al., 2002]. It can also act independent of the IGFs by

interacting with $\alpha_5\beta_1$ integrin to cause cell adhesion and migration [Firth and Baxter, 2002]. Its exact role in colon cancer is not clearly known.

IGFBP-2

IGFBP-2 is up-regulated in different pathological and unphysiological situations like trauma, certain tumours and starvation. Transgenic mice over- expressing IGFBP-2 show significantly reduced body weight gain, demonstrating that IGFBP-2 is a negative regulator of normal somatic growth, most probably by sequestering the IGFs from their receptors [Hoeflich et al., 2001]. IGFBP-2 inhibits cell proliferation in several IGF-responsive colon carcinoma cell lines, but, in contrast, Miraki-Moud et al have shown that IGFBP-2-overexpressing Caco-2 cells grow at a faster rate, indicating that the IGFBP-2 stimulates cell proliferation. Therefore, the IGFBP-2 can either inhibit or potentiate cancer cell growth under different circumstances.

In human colon cancer, IGFBP-2 mRNA is often over-expressed, and circulating IGFBP-2 is often elevated [Kim et al., 2002c]. The IGFBP-2 level often reflects the tumour grade and the stage of the disease [Hoeflich et al., 2001]. In one study, IGFBP-2 mRNA levels were increased 4- to 8-fold in patients with colon cancer compared to controls. Patients with Dukes stage C disease had the highest levels of IGFBP-2 mRNA. Therefore, the IGFBP-2 may be implicated in colon cancer metastases and prognosis. Its sensitivity as a tumour marker increases when used in combination with carcinoembryonic antigen (CEA) [Renehan et al., 2000a]. Serum IGFBP-2 may have an adjunct role in cancer surveillance in patients with colorectal cancer.

IGFBP-3

Like IGF-I, most of the IGFBP-3 found in the circulation is produced by the liver under the influence of growth hormone. It has both IGF-I inhibiting and potentiating actions. High levels of circulating IGF-I, and particularly low levels of IGFBP-3, are associated independently with an elevated risk of large or tubulovillous /villous colorectal adenoma and cancer [Giovannucci et al., 2000]. The IGFBP-3 acts independent of IGFs by enhancing the p53-dependent apoptotic response of colorectal cells to DNA damage [Williams et al., 2000] [Howarth, 2003].

IGFBP-5

IGFBP-5 expression is up-regulated by anti-proliferative agents, such as retinoic acid and vitamin D-related compounds. The exact role of the IGFBP-5 in colorectal cancer is not known. Studies involving prostate cancer cell lines show that the IGFBP-5 stimulates growth via IGF-dependent and -independent mechanisms [Schneider MR et al., 2002]. But it inhibits the growth of osteosarcoma and cervical cancer cell lines [Schneider MR et al., 2002]. It has both stimulatory and inhibitory effects on breast cancer cells, depending upon the stage[Butt et al., 2003;Perks and Holly, 2000]. It means that it has a complex role in cancer, which needs further research.

IGFBP-6

The IGFBP-6 has a high affinity for IGF-II [Oh et al., 2001]. The IGFBP-6 was found to inhibit both HT-29 and Caco-2 cell proliferation, by binding to endogenously produced IGF-II, thereby preventing IGF-II from interacting with the

IGF-I receptor, to stimulate cellular proliferation by autocrine mechanism [Kim et al., 2002c].

IGFBP-7 (IGFBP-rP-1)

IGFBP-7 is also known as 'mac 25', and it shows 20-25% similarity to other IGFBPs [Oh et al., 1996]. The IGFBP-7 specifically binds to both IGF-I and IGF-II. In comparison with IGFBP-3, the IGFBP-7 has at least a 5- to 6- fold lower affinity for IGF-I, and a 20- to 25- fold lower affinity for IGF-II. The exact role of this binding protein in colon cancer is not clear.

1.2.9 Summary

The IGF system has a complex role in the development and progress of colorectal cancer. This system is essential for human day-to-day tissue regeneration. The biological activity of IGF is determined by the integrated actions of circulating IGF-I and IGFBP, and by local production of IGF, IGFBP and IGFBP protease. So far *in vitro* and *in vivo* studies, as well as population-based studies, have shown that increased IGF-I and -II may be related to the development of colon cancer. Since the association is not clearly proven consistently, it appears that the ligands may play an adjuvant role rather than primarily inducing carcinogenesis. When activated, the IGF-IR not only stimulates the synthesis of RNA and DNA, cell proliferation, differentiation and increases cell survival, but it also increases angiogenesis. All IGFBPs bind to free IGFs and inhibit their action on IGF-IR, which is vital for carcinogenesis. Thus, IGF-IR is more important than any other factor because it mediates the growth of cancer. IGF-IR antagonism using monoclonal antibody, when combined with chemoradiation, produces an increased cytotoxic response, which may be useful in clinical situations. Manipulation of the IGF-IR may help to control

several cancers, and this needs to be studied properly. Loss of imprinting of the IGF-II gene is responsible for at least some colorectal cancers. Thus, it may be possible after further study to cure this LOI by insertion of correct gene copy. We do not have enough evidence to recommend the use of IGFBPs in clinical therapeutic trials for cancer. Serum IGFBP-2 reflects tumour load, so it could be used in cancer surveillance in patients with colorectal cancer, after further large studies. Its sensitivity as a tumour marker increases when used in combination with CEA. IGFBP-3 is the main binding protein which carries and binds more than 90% of the IGFs in circulation. Manipulation of the IGF-IR may help to control several cancers, which have to be studied extensively. Some of the binding proteins like IGFBP-5 and -7 have both stimulatory and inhibitory effects under different circumstances. If the future studies show the exact circumstances under which they inhibit cancer growth, it can be utilised to arrest cancer growth. As stated in the meta-regression analysis by Renehan et al, case control studies might have overestimated the magnitude of association between IGF and cancer. The method of collection is a source of heterogeneity. It has to be standardised so that all available data can be compared, otherwise the results of the studies done by different individuals will show different results. Much of our current knowledge is from *in vitro* studies. There is not much information available regarding the usage of these proteins in the prevention of cancer *in vivo*. There is, therefore, a need for further *in vivo* studies to enable us to manipulate these binding proteins, to prevent and control various cancers. As discussed earlier in this chapter, IGFBP-4 will be described separately in the next chapter.

1.3 Biology of IGFBP-4

Among the IGFBPs, IGFBP-4 is the smallest [Zhou et al., 2003] and it exists in two forms: non-glycosylated (24 kDa) and N-glycosylated forms (28 kDa) [Cheung et al., 1991;Ceda et al., 1991;Kelley et al., 1996]. It occurs in most biological fluids [van Doorn et al., 2001]. By binding to IGF-I and IGF-II with similar affinities, it inhibits their actions under almost all *in vitro* and *in vivo* conditions. The glycosylation of IGFBP-4 does not affect its binding to IGF-I [Chelius et al., 2001]. The liver is the main source of serum IGFBP-4 [Mazerbourg et al., 2004], but it is abundantly expressed by many tissues, including adrenals [Ilvesmaki et al., 1993], Leydig cells and interstitial connective tissue of testis [Zhou and Bondy, 1993], and the developing embryo, with the notable exceptions of the spinal cord, specific cartilage groups and the thymic cortex [Cerro et al., 1993]. The serum level of intact IGFBP-4 is low and is influenced by vitamin D and the parathyroid hormone [Honda et al., 1996]. The amount of serum IGFBP-4 shows a positive correlation with age [Honda et al., 1996]. IGFBP-4 is expressed by several cancer cell lines [Bostedt et al., 2001;Ceda et al., 1991;Yi et al., 2001]. Overexpression of IGFBP-4 has been shown to reduce the growth of some cancers [Damon et al., 1998].

1.3.1 Structure and binding characteristics of IGFBP-4

The gene that encodes IGFBP-4 is located in the chromosome region 17q12-q21.1 [Tonin et al., 1993]. IGFBP-4 protein has 237 aminoacids and 20 cysteines [Hwa et al., 1999]. When compared to other IGFBPs, IGFBP-4 has two extra cysteine residues in the variable region encoded by exon 2 [Qin et al., 1998]. The IGFBP-4 has three domains [Mazerbourg et al., 2004] namely N-terminal, C-terminal and

central domain. The binding domain of IGF-I and IGF-II involves a hydrophobic motif (Leu (72)-Met (80)), located in the distal part of the conserved N-terminal region. N-terminal Cys residues (Cys9 and Cys12) are more critical than C-terminal Cys residues (Cys17 and Cys20) in affecting the IGF-I and IGF-II binding. The C terminal fragments of IGFBP-4 do not bind to IGFs, but loss of this fragment decreases the affinity for the IGFs. A study [Qin et al., 1998] showed that deletion of Leu-Ser resulted in loss of both IGF-I and IGF-II binding, and substitution of Histidine to Proline abolished both IGF-I and IGF-II binding. This evidence shows that IGFBP-4 has a single binding site for both IGF-I and -II.

1.3.2 Biological actions of IGFBP-4

IGFBP-4 acts by binding to IGF-I and IGF-II and modulating their biological effects [Honda et al., 1996]. IGFBP-4 also has actions which are independent of IGF-I and IGF-II. Local and systemic administrations of IGFBP-4 have different effects. Single local administration of IGFBP-4 over the right parietal bone of mouse inhibits IGF-I-induced bone formation, whereas systemic administration of IGFBP-4 alone increases serum levels of bone formation markers [Miyakoshi et al., 1999]. Overexpression of IGFBP-4 in transgenic mice resulted in decreased growth of thymus [Zhou et al., 2004], a reduction in weight of smooth muscle-rich tissues, including bladder, intestine, aorta, uterus, and stomach, without any change in total body weight [Schneider et al., 2000;Wang et al., 1998a]. These indicates that IGFBP-4 is a functional antagonist of IGF-I action on smooth muscle *in vivo* [Wang et al., 1998a]. IGFBP-4 excess also inhibits cell proliferation and stimulates apoptosis in lymphoid tissues, but it does not affect lymphocyte development [Zhou et al., 2004]. Exogenous IGFBP-4 decreased thrombin-induced DNA synthesis of human aortic vascular smooth muscle cells by 64%, whereas anti-IGFBP-4 antibody

potentiated thrombin-induced DNA synthesis [Anwar et al., 2000]. This data suggests that down-regulation of IGFBP-4 expression in vascular smooth muscle, may play a critical role in vascular growth response in normal and diseased states, by increasing the bioavailability of IGF-I [Anwar et al., 2000]. In addition to its anti-proliferative action, the IGFBP-4 has been correlated to differentiation of cancer cells [Shen and Singh, 2004].

IGF-dependent action

It has been shown that IGFBP-4 inhibits IGF-I-stimulated DNA synthesis in both cancerous and non-cancerous cells [Hsieh et al., 2003], but IGFBP-4 has no effect on cell proliferation induced by analogs of IGF-I or IGF-II, which exhibit more than 100-fold reduced affinity for binding to IGFBP-4. The inhibitory action of IGFBP-4 on IGF-I-induced DNA synthesis occurs in a concentration-dependent manner, as evidenced in vascular smooth muscle cells [Duan and Clemmons, 1998]. IGFBP-4 inhibits IGF action by preventing the binding of ligand to the IGF-IR [Mohan et al., 1995].

IGF-independent action

The IGF-independent action of IGFBP-4 is not well understood. In a study [Wright et al., 2002], when granulosa cells of ovary were incubated with gonadotropins and a IGF-IR blocker (αIR3), the IGFBP-4 continued to exert potent inhibitory effects, even when the action of endogenous IGF was removed from the system, demonstrating that its actions are independent of IGF binding. In an another study [Perks et al., 1999], IGFBP-4 caused marked ($P < 0.01$) inhibition of ceramide-induced apoptosis in Hs578T breast cancer cells. In IGF-insensitive Isreco-1 cells, reduced colony formation, but not cell proliferation and migration, was found while IGFBP-4 was overexpressed.

1.3.3 Factors controlling IGFBP-4 expression

Gene

It has been found that the 1.4 kilobase pair 5' flanking region of the IGFBP-4 gene containing cis elements is required for the regulation of the IGFBP-4 gene [Dai et al., 1997]. In a recent study, a new binding site known as the Sp3 site has been shown to influence the expression of IGFBP-4 gene, particularly in Caco-2 cells [Shen and Singh, 2004;Demori et al., 2000].

Trauma and tissue regeneration

Cerebral contusions increase cortical expression of IGFBP-4 mRNA levels at the contusion site, and along the ipsilateral cortex [Nordqvist et al., 1997]. It has been shown that serum IGFBP-4 begins to increase 12-24 hours after partial hepatectomy [Demori et al., 2000], consistent with the increase in corresponding mRNA. These suggest a regulatory mechanism that modulates IGF activity during tissue regeneration.

Proteolysis

IGFBP-4 is subject to proteolytic cleavage by several proteases [Byun et al., 2000;Durham et al., 1995]. One of the IGFBP-4 proteases, also known as pregnancy-associated plasma protein A (PAPP-A) is an important regulator of local IGF bioavailability and cell growth [Chen et al., 2002]. A study conducted in our department on 18 samples of resected colon cancer specimens, showed that PAPP-A is expressed by both malignant and non-malignant colon [Mark Davies, 2004]. The study also showed that malignant colon expresses less IGFBP-4 than the normal colon. This could be due to either increased proteolysis in the malignant colon or

decreased transcription of its gene. Colo-205 secretes large quantities of IGFBP-4 but it remains responsive to IGF-I. Analysis of the conditioned medium revealed cleavage of IGFBP-4 into less active fragments, which have less affinity for IGFs, which supports the argument that proteolytic cleavage of IGFBP-4 which controls the bioavailability of IGFs [Mark Davies, 2004]. Proteases cleave IGFBP-4 into two fragments of approximately 18 and 14 kDa, both of which have poor affinity for IGFs [Kelley et al., 1996;Remacle-Bonnet et al., 1997]. Sequence analysis of the 14-kDa carboxyl-terminal half of IGFBP-4 suggested cleavage after methionine at position 135 of the mature protein [Conover et al., 1995a]. *In vivo* and *in vitro* experiments have shown that lysosomal protease cathepsin D can also mediate proteolysis of IGFBP-4 [Braulke et al., 1995].

Calorie intake

In a study on rats' stomach IGF-I and IGFBP-4 mRNA levels increased significantly ($P<0.05$) when the calorie intake was restricted [Hallberg et al., 2000]. There were no changes in colonic IGFBP-4 mRNA levels in aged and long-term calorie restricted rats. It shows that the stomach attempts to preserve IGF activity by increasing local expression of IGF-I and IGFBPs [Hallberg et al., 2000]. A separate study showed that systemic levels of IGFBP-4 did not change with a low-fat diet [Ngo et al., 2003].

Hormones and growth factors

It appears that several hormones and growth factors may influence the level of IGFBP-4 locally, but not enough to alter the levels systemically. They include IGF-I [Price et al., 1995],IGF-II [Feliers et al., 1999], oestrogen [Sheikh et al., 1993], IL-1β [Street et al., 2003], and IL-6 [Street et al., 2003;Fernandez-Celemin and Thissen,

2001]. IGFBP-4 is apparently not regulated in response to TNF-α, PDGF, TGF-β or the cAMP agonist, forskolin in multiple myeloma cells [Feliers et al., 1999]. IGFs may regulate their own availability through proteolytic degradation of IGFBP-4 [Noll et al., 1996] as attachment of IGFs to IGFBP-4 result in enhancement of proteolysis [Clemmons, 1993]. IGFBP-3 is reported to inhibit IGFBP-4-degrading proteinase activity, and binding of IGFs or glycosaminoglycans to IGFBP-3 may induce conformational changes in the binding protein, causing disinhibition of the proteinase [Fowlkes et al., 1995].

Both growth hormone status and pharmacological doses of glucocorticoids do not affect plasma IGFBP-4 [van Doorn et al., 2001] although, apparently, a weak positive relationship exists between the plasma IGFBP-4 and parathormone [van Doorn et al., 2001]. Neither hypothyroidism nor hyperthyroidism influence circulating IGFBP-4 levels [van Doorn et al., 2001] but, in a study involving rat hepatocytes, the IGFBP-4 levels were increased in the presence of tri-iodothyronine [Demori et al., 1997a]. Retinoic acid and thyroid hormone act synergistically and increase IGFBP-4 expression in cultured rat hepatocytes [Demori et al., 1997b;Demori et al., 1997a;Demori et al., 2004].

1.3.4 IGFBP-4 in cancer

There is accumulated evidence showing a link between IGFBP-4 and a variety of cancers. Several cancer cell lines, including multiple myeloma [Feliers et al., 1999], neuroblastoma [Babajko and Binoux, 1996;Babajko et al., 1997], small cell and non-small-cell lung cancer [Wegmann et al., 1993;Pavelic et al., 2002a;Bostedt et al., 2001], mesothelioma [Hodzic et al., 1997], gastric cancer [Yi et al., 2001;Guo et al.,

1993], thyroid cancer [Bachrach et al., 1995], breast cancer [Sheikh et al., 1992;Sheikh et al., 1993;Pekonen et al., 1992;McGuire, Jr. et al., 1992], prostate cancer [Conover et al., 1995b;Srinivasan et al., 1996] and colon cancer [Michell et al., 1997b], have been reported to express IGFBP-4. The role of IGFBP-4 in cancer is discussed below under three headings, namely *in vitro*, *in vivo* and population-based studies.

1.3.5 The role of IGFBP-4 from *in vitro* studies

Gastrointestinal cancers

The majority of colon cancers studied were found to express IGFBP-4 [Dai et al., 1997;Srinivasan et al., 1996]. In an *in vitro* study, Diehl et al [Diehl et al., 2004] overexpressed IGFBP-4 in colon cancer cells using murine IGFBP-4 cDNA. Overexpression of IGFBP-4, in IGF-insensitive colon (Isreco) cells, decreased the colony formation alone, without any effect on cell proliferation and migration, but in IGF-dependent LS1034 cells, it decreased proliferation, migration and colony formation, although IGF-II partly restored the first two parameters. In another IGF-sensitive cell, Isreco-2, which lacks endogenous IGF expression, the colony formation was decreased by IGFBP-4. In a different study [Park et al., 1996], Caco-2 cells were transfected with a human IGFBP-4 cDNA construct, and they exhibited a 60% increase in IGFBP-4 mRNA, and secreted twice as much IGFBP-4 protein as in controls. The IGFBP-4 overexpressing cells proliferated at only 25% the rate of control cells in serum-free medium, and there was a 70% increase in expression of sucrase-isomaltase [Park et al., 1996]. IGFBP4 gene expression plays an important role in the transition from proliferation to differentiation in colon cancer cell line Caco2 [Shen and Singh, 2004]. IGFBP-4 expression in Caco-2 cells correlated well

with cell differentiation [Hoeflich et al., 1996] and a significant up-regulation of IGFBP-4 expression occurred on spontaneous differentiation in culture [Dai et al., 1997]. Anti-sense inhibition of IGFBP-4 mRNA confers a growth advantage to the cells in response to endogenous and exogenous IGFs [Dai et al., 1997]. Singh [Singh et al., 1994a] studied the inhibitory role of endogenous IGFBP-4 on human colon cancer cell line HT-29 cells. Both basal and the IGF-stimulated growth of HT-29 cells were significantly increased over control values in the presence of IGFBP-4 antibody, suggesting that endogenous IGFBP-4 is a potent inhibitor of the mitogenic effects of endogenous and exogenous IGFs [Singh et al., 1994a]. Singh et al also transfected colon cancer cells with sense and anti-sense cDNA fragments of human IGFBP-4. Basal and the IGF-I-stimulated growth of the anti-sense cells were significantly higher than that of control and sense cells. The basal and the IGF-I stimulated growth of the sense cells was not significantly different from that of the control cells, suggesting that overexpression of IGFBP-4 was not inhibitory to the growth of the HT-29 cells.

HT29-D4 cells secreted IGF-II which became totally complexed to IGFBP-2, IGFBP-4 and IGFBP-6, and ~15% of IGFBP-4 was associated with the extracellular matrix [Remacle-Bonnet et al., 1997]. IGFBP-4 proteolysis by cell-bound plasmin can promote autocrine/paracrine IGF-II bio-availability in colon-cancer cells [Remacle-Bonnet et al., 1997]. Corking and co-worker [Corkins et al., 2002] studied the effect of carbohydrate on expression of IGFBP-4 by HT-29 cell. The cells were grown in either glucose or galactose (glucose free) medium. Cells grown in galactose medium showed low IGFBP-4 levels until the cells approached confluence, at which point the levels increased significantly, while the cells grown in glucose medium

showed increasing IGFBP-4 levels with increasing cell numbers, except for a transient decrease at confluence. HT-29 cells, when treated with retinoic acid, had dose-dependent increases in IGFBP-4 and reduced IGF-II expression. In a different study [Akagi et al., 1998], HT29 cells were treated with IGF-I for various time periods, which resulted in increased VEGF mRNA expression . When the activity of IGFBP-4 was blocked, it did not significantly influence the effect of IGF-I induction of VEGF mRNA in the HT29 cells [Akagi et al., 1998] .

A previous study conducted in our department to assess the expression of IGFBP-4 protein in malignant and non-malignant colonic tissue in 18 patients, showed a significantly lower amount of IGFBP-4 in colon cancers than in non-malignant colon in 16 patients ($P<0.05$) [Mark Davies, 2004], which was confirmed by both Western blot as well as immunohistochemistry [Mark Davies, 2004]. This may be due to increased protease activity of transformed cells. Expression of IGFBP-4 has also been found in most gastric carcinoma cell lines [Guo et al., 1993;Yi et al., 2001] but it is important to distinguish between increased expression at the mRNA level and the concentration of the protein. Regrettably, this is often overlooked as the functional aspects of IGFBP-4 are dependent on the protein.

Neuroblastoma and glioma

It was found that rat neuroblastoma cell line secreted both glycosylated and non-glycosylated forms of IGFBP-4, and IGF-II treatment decreased the levels of both form of IGFBP-4 in the culture media [Ceda et al., 1991]. This decrease in the expression was dose-dependent and could be blocked by the addition of IGF-I to the cells. Although both IGF-I and IGF-II affected the amount of the IGFBP-4, neither peptide affected the expression of the mRNA of 24K IGFBP-4. Overexpression of

intercellular communication gap junction gene connexin 43 in glioma cells, resulted in decreased cellular proliferation due to higher production of IGFBP-4 [Bradshaw et al., 1993]. In a different study, IGFBP-4 inhibited the binding of [^{125}I] IGF-I by its receptors, and blunted the stimulation of [^{3}H] thymidine incorporation by IGF-I. In B104 cells, a rat neuronal cell line, IGFBP-4 is the predominantly secreted IGFBP-4 [Cheung et al., 1994]. Exposure of B104 monolayer cultures to dexamethasone, reduced native IGFBP-4 abundance to <10% of that in control medium by 48 hours [Cheung et al., 1994]. This was due to increased IGFBP-4 activity induced by dexamethasone. These findings suggest a role for IGFBP-4 in neural and neuroblastoma cell function [Cheung et al., 1991].

Lung cancer

Not all lung cancers have been found to express IGFBP-4 [Pavelic et al., 2002a]. In a study involving 69 human lung carcinoma tissues >50% (35/69) of samples were positive for IGFBP-4 mRNA [Pavelic et al., 2002a]. In a cell culture experiment, IGFBP-4 levels diminished with increasing concentrations of IGFs [Bostedt et al., 2001] in the culture media of non small cell lung cancer cells, whereas IGFBP-4-specific mRNA was not changed by IGF-I or IGF-II. Either IGFs may activate an IGFBP-4-specific metalloprotease present in culture media, or the binding of IGFs to IGFBP-4 may increase the susceptibility of IGFBP-4 to proteolytic degradation [Noll et al., 1996].

Endocrine cancers

Both human thyroid follicular carcinoma and papillary carcinoma cells produce IGFBP-4 [Bachrach et al., 1995;Isozaki et al., 1996;van der Laan et al., 1995]. In a cell culture experiment involving two human thyroid follicular carcinoma cell lines

(FTC-133 and FTC-236 cells), IGFBP-4 secretion was increased in the presence of thyroid stimulating hormone, forskolin, and epidermal growth factor, and was reduced by tetradecanoylphorbol acetate [Bachrach et al., 1995]. Nodular and bilateral hyperplasia of the adrenal gland, as well as pheochromoctyomas showed higher expression of IGFBP-4 mRNA, while non-functional adrenal carcinomas and normal adrenals expressed less IGFBP-4 mRNA [Ilvesmaki et al., 1998].

Breast cancer

The relationship between IGFBP-4 expression and hormonal receptors in breast cancer is unclear. Figueroa and co-workers studied 40 primary breast tumors [Figueroa et al., 1993] and found that all of them expressed IGFBP-4 mRNA, and the expressions were higher in oestrogen receptor (ER)-positive specimens. Another study on 80 breast cancer specimens showed that IGFBP-4 was positively correlated with both estrogen and progesterone receptors, and inversely correlated with synthetic-phase (S-phase) fraction [McGuire et al., 1994], which reflects tumour cellular proliferation. In contrast to these two studies, another study on 47 cancer specimens failed to find any relation between ER content and IGFBP-4 levels [Pekonen et al., 1992]. In an experiment involving MCF-7 breast cancer cells, IGFBP-4 had no effect on IGF-I-induced DNA synthesis [Chen et al., 1994] but, in Hs578T cells IGFBP-4 caused marked ($P < 0.01$) inhibition of ceramide-induced apoptosis [Perks et al., 1999].

The expression of IGFBP-4 in breast cancer is modulated by various hormones. Treatment of MCF-7 cells with an oestrogen, 17β-estradiol, resulted in increased IGFBP-4 gene expression (>3-fold) and protein secretion (>6-fold). Further evidence showed that anti-estrogens ICI 164384 and ICI 182780 decreased mRNA levels of

IGFBP-4 [Coutts et al., 1994;Pratt and Pollak, 1993]. In N-nitrosomethylurea-induced rat mammary tumour, IGFBP-4 mRNAs decreased with ovariectomy and increased with hormone repletion. Another anti-oestrogen, tamoxifen, significantly reduces the levels of IGFBP-4 protein in the conditioned medium of MCF-7 cells [Pratt and Pollak, 1993]. A small study on 8 patients showed an increase in serum IGFBP-4 levels after tamoxifen [Gronbaek et al., 2003], which may reflect the systemic effect of tamoxifen.

In a different study, medroxyprogesterone acetate treatment resulted in a time- and dose-dependent decrease in IGFBP-4 mRNA, but RU 486 alone had little or no effect [Coutts et al., 1994]. A synthetic progestin, Org 2058, but not dexamethasone, inhibited IGFBP-4 expression. In a separate study, IGFBP-4 levels were slightly decreased in response to high doses of human chorionic gonadotropin [Huynh, 1998].

It has been shown that retinoic acid increases IGFBP-4 levels in conditioned medium in MCF-7 breast carcinoma cell line [Adamo et al., 1992]. In contrast, another study showed that retinoic acid reduced transcriptional activity of the IGFBP-4 gene [Chambery et al., 1998]. Retinoic acid and oestrogen increased IGFBP-4 mRNA levels in many ER-positive cell lines, and the effect of oestrogen and retinoic acid in combination was additive [Sheikh et al., 1993]. Although both of them individually enhanced IGFBP-4 mRNA levels in ER-positive T47D cells, their effect in combination was antagonistic in this cell line [Sheikh et al., 1993].

Osteosarcoma and multiple myeloma

Normal osteoblasts secrete IGFBP-4 as well as an IGF-dependent IGFBP-4 protease [Durham et al., 1995]. Therefore, the IGF system can influence bone growth by altering the levels of IGFBPs, and thereby influencing free IGFs. Fully tumourigenic osteosarcoma cells do not express IGFBP-4 as well as an IGF-dependent IGFBP-4 protease [Durham et al., 1995]. Human myeloma cells express IGFBP-4 [Feliers et al., 1999]. In late stages of myeloma, there is increased bone destruction and decreased bone formation, which causes osteolytic lesions. This may be due to multiple myeloma cells secreting IGFBP-4, which inhibits IGF-I-stimulated bone formation by adjacent normal osteoblasts [Feliers et al., 1999].

Prostate cancer

Prostate carcinoma cells secrete IGFBP-4 [Drivdahl et al., 1995;Conover et al., 1995b], as well as a general IGFBP protease and cathepsin D, both of which are capable of hydrolyzing all endogenous IGFBPs and, thus, modifying IGF-I action in prostatic cells [Conover et al., 1995b]. In one study, IGFBP-4 was overexpressed by transfecting malignant M12 prostate epithelial cells with plasmid containing IGFBP-4 [Damon et al., 1998]. Consequently, IGF-induced proliferation was reduced in the IGFBP-4 transfected cells compared with control cells ($P \leq 0.01$). Colony formation in soft agar was inhibited for 14 days ($P \leq 0.01$). As IGFBP-4 has been suggested to inhibit cell growth, the reduction of IGFBP-4 expression should increase the availability of free IGFs, which would increase cell growth. In reality, the results were different. In a study [Drivdahl et al., 2001], when IGFBP-4 expression was inhibited with anti-sense cDNA in two prostate tumor cell lines, ALVA-31 and M12, both transfected lines proliferated more slowly in monolayer culture than parental

controls. Colony formation in soft agar was strongly inhibited in both cases. Apoptosis induced by the topoisomerase inhibitor, etoposide, was also enhanced in transfected cells. The results may be due to involvement of other binding proteins which influence cell growth.

1.3.6 The role of IGFBP-4 from *in vivo* studies

When compared to *in vitro* studies the numbers of *in vivo* studies which demonstrate the effect of IGFBP-4 in cancer are much fewer and less well standardised.

Hepatocellular carcinoma

IGFBP-4 expression in pre-neoplastic and neoplastic lesions is not the same. Immunohistochemical studies showed increased expressions of IGF-I and IGFBP-4 in pre-neoplastic lesions [Scharf et al., 2000]. Hepatocellular carcinoma arising in this model showed decreased expressions of IGF-I and IGFBP-4. The altered gene expression in glycogen-storing preneoplastic hepatic foci, especially the up-regulation of IGF-I and IGFBP-4 with the down-regulation of IGFBP-1, resemble the insulin-dependent regulation of these components in normal rat hepatocytes.

Prostate cancer

Damon [Damon et al., 1998] transfected prostate cancer cells with IGFBP-4 gene, and injected these cells subcutaneously into male athymic/nude mice. There was a marked delay in tumor formation in animals receiving IGFBP-4 transfected cells when compared with controls ($P \leq 0.01$). In a reverse experiment, IGFBP-4 expression was inhibited with anti-sense cDNA in prostate tumor cell line, M12, it also markedly reduced the rate of tumor formation and growth in male athymic nude

mice [Drivdahl et al., 2001]. These studies demonstrated that the *in vivo* effect of altered expression of IGFBP-4 is complex and is under the influence of several unknown factors.

1.3.7 The role of IGFBP-4 from population and clinical studies

In a case-control study [Ng et al., 1998] involving 63 breast cancer patients, decreasing serum levels of IGFBP-4 ($p < 0.01$) were significantly associated with an increasing numbers of progesterone receptors in the tumour. IGFBP-4 was significantly ($p < 0.01$) associated with the risk of breast cancer.

In one study, the IGFBP-4 levels in the circulation did not show any difference in most of the cancer patients with solid tumours, although several children with acute lymphoblastic leukaemia showed increased plasma IGFBP-4 levels [van Doorn et al., 2001].

1.4 Hypothesis

The hypothesis of the study was that gene construct containing insulin-like growth factor binding protein - 4 (IGFBP-4) cDNA, when administered by local injection, would increase the expression and secretion of IGFBP-4 protein by colorectal cancer cells, neighbouring cells and skeletal muscle cells. As it sequesters insulin-like growth factors, the IGFBP-4 may inhibit or decrease all IGF induced functions, including cellular proliferation and anti-apoptosis. The growth of colon cancer cells when deprived of IGFs may be inhibited. So when the gene is administered after the induction of tumour, IGFBP-4 may delay further tumour growth and increase the apoptosis, leading to reduction in the size of the tumour. When the gene construct is administered at an early stage, IGFBP-4 may prevent or delay tumour formation from cancer cells.

1.5 Objectives

The objectives of the study presented in this thesis was to treat the subcutaneously induced cancers with plasmids containing IGFBP-4 cDNA, and assess the effect of IGFBP-4 gene therapy on tumour characteristics, IGF system expression, apoptosis and proliferation of cancer cells.

The first step of this project was multiplication and purification of plasmid containing IGFBP-4 cDNA and confirmation of its sequence, and then to induce subcutaneous cancers by inoculating nude mice with HT-29 cells, so that the plasmid containing IGFBP-4 cDNA could be administered by local injection either on day '0' or after the development of palpable tumour.

Once attaining a reasonable size, the subcutaneous tumours were assessed for cellular proliferation, apoptosis and expression of components of IGF system including IGFBP-4. Finally Bax and Bcl-2 levels were determined to explore the mechanism of apoptosis.

1.6 Research overview

As shown in the flow chart (figure 1.3) plasmids were extracted and HT-29 adenocarcinoma cells were cultured simultaneously. Once sequencing confirmed the presence of IGFBP-4 cDNA insert in the plasmid, subcutaneous cancers were induced in nude mice. The experiments were performed in two models. In the first model (late gene transfer), there were two groups and the therapeutic effect of IGFBP-4 was assessed. In this model, the gene construct was administered once the tumour was established. In the second model (early gene transfer = preventive model), there were three groups and the role of IGFBP-4 in prevention of cancer was assessed. So the plasmids were administered even before the cancer was established, i.e. the cancer cells and plasmids were administered together. Three to four weeks following IGFBP-4 induction, the tumours were harvested and assessed for, mitosis, apoptosis, various proteins including IGF-IR, IGFBP-4, Bcl-2 and Bax by various laboratory based methods.

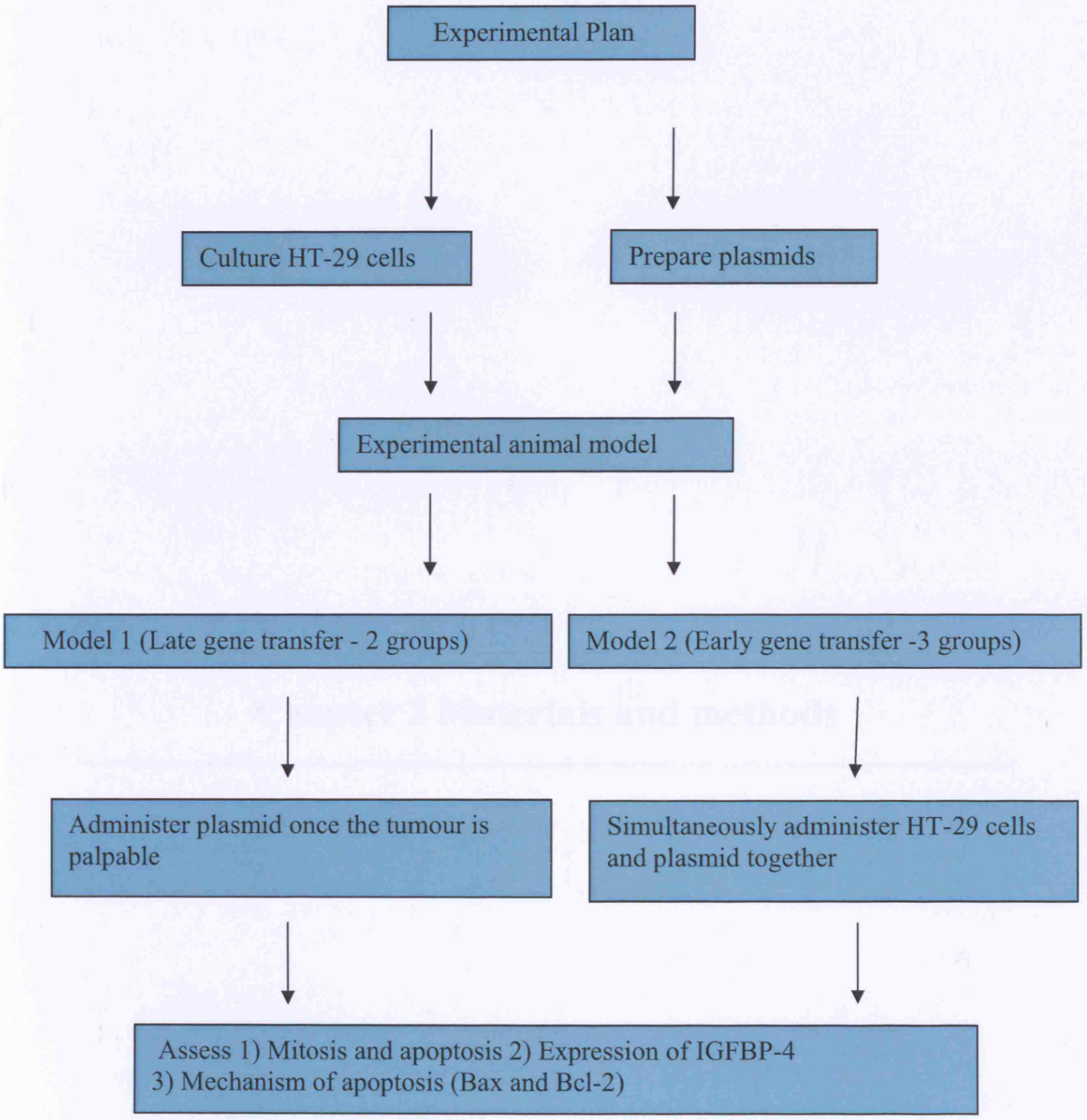


Figure 1.3 Overview of research planning. HT-29 cells were cultured and plasmids were prepared simultaneously. Then the effects of the plasmids were assessed on colon cancer cells *in vivo* at two different time periods in relation to mitosis, apoptosis, and expression of IGFBP-4, Bax and Bcl-2 proteins.

Chapter 2 Materials and methods

2.1 Preparation of gene construct

2.1.1 Gene construct

Mammalian expression vector pcDNA3 (Invitrogen, Carlsbad, CA, USA) containing myosin light chain enhancer was used in this study, in which IGFBP-4 cDNA was inserted between *Kpn* I and *Eco*R I restriction sites down stream of cytomegalovirus (CMV) promoter. This plasmid was kindly provided as a gift by Mr Steve Coe, Molecular Tissue Repair Unit, Royal Free and University College Medical School, London.

pcDNA 3 (figure 2.1) is an artificial 5.4 kb vector, with a double stranded circular DNA containing 5446 nucleotides, designed for high level stable expression in most mammalian cells. It contains CMV intermediate-early promoter for high-level expression, neomycin resistance gene for selection of stable cell lines and two promoters (T7 and SP-6). The presence of the ampicillin gene provides resistance to ampicillin. Figure 2.2 shows the genetic sequence of pcDNA3. The plasmid and the foreign DNA that needs to be studied can be cut by restriction endonuclease, producing intermediates with sticky and complementary ends. The two intermediates recombine by base-pairing and are linked by the action of an enzyme called DNA ligase. Thus a new plasmid containing the foreign DNA as an insert is obtained. The plasmids can be easily transformed with bacteria Escherichia Coli (E.Coli).

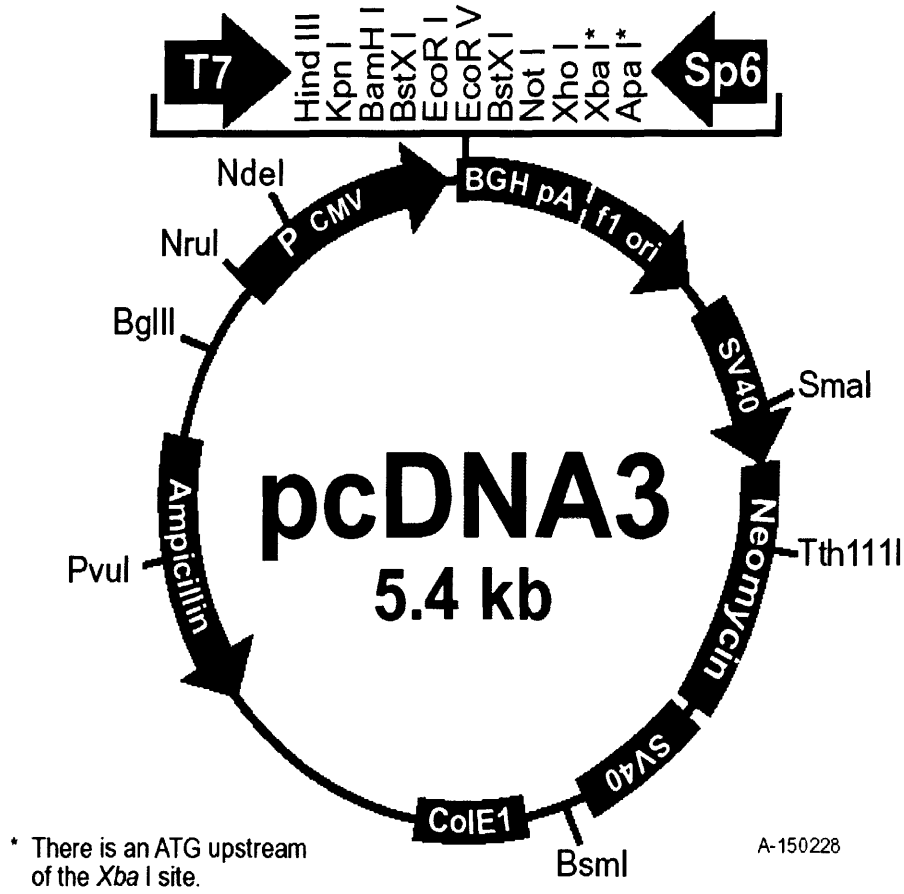


Figure 2.1 Structure of mammalian expression vector pcDNA 3. It is a 5.4 kb vector with a double stranded circular DNA containing 5446 nucleotides. It contains cytomegalovirus promoter for high-level expression, neomycin resistance gene for selection of stable cell lines and two promoters (T7 and SP-6). (Reproduced from Invitrogen, USA website)

pcDNA3
hCMV immediate early promoter
Polylinker
Primer binding sites
Bovine Growth Hormone polyA

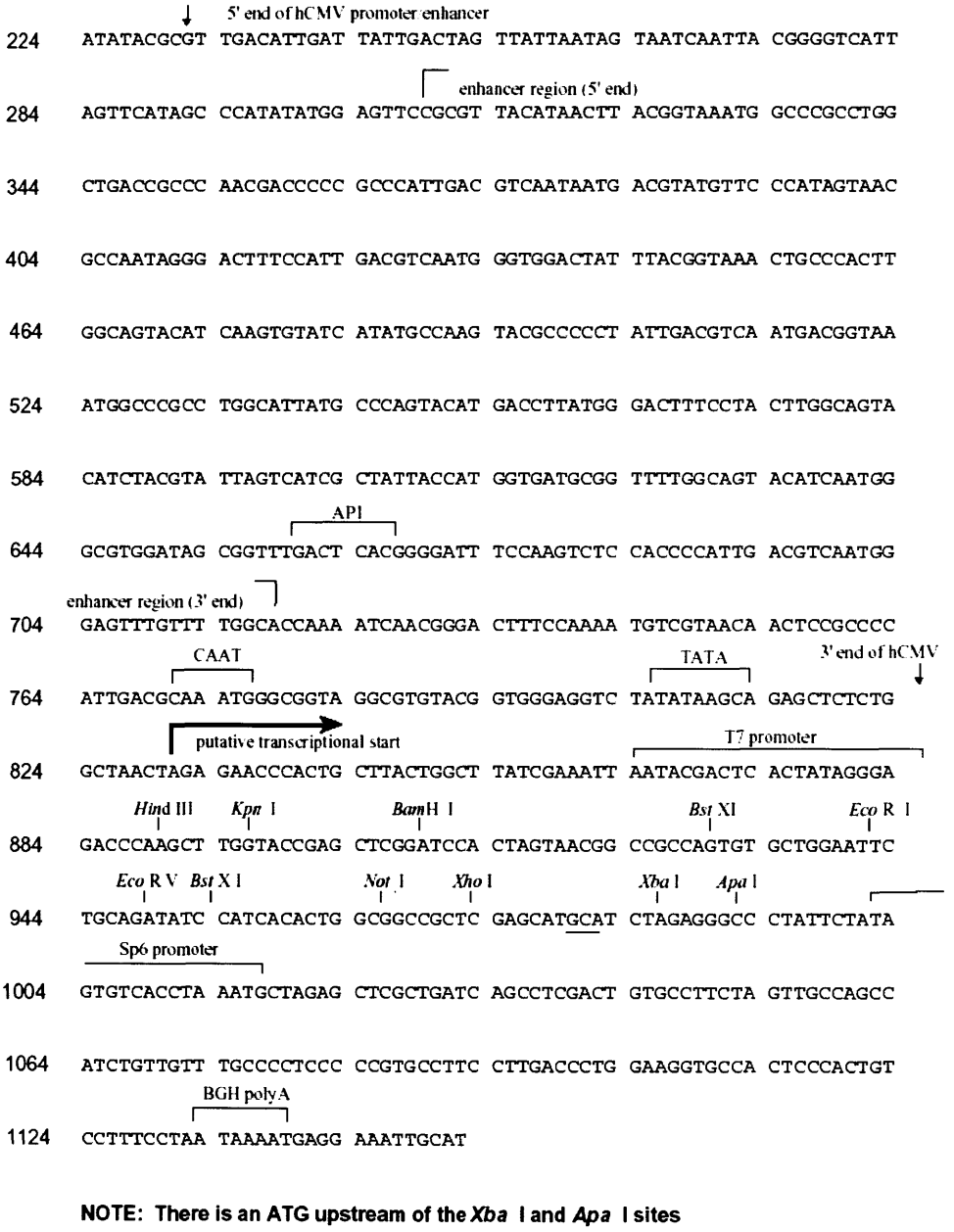


Figure 2.2 Genetic sequence of mammalian expression vector pcDNA 3

(Reproduced from Invitrogen, USA website)

2.1.2 Transformation of vector

The plasmids (vector and vector with IGFBP-4 cDNA) were introduced into competent cells to multiply them. Competent cells are in such a state in which bacteria are able to take up foreign DNAs. Transformation is a process of introducing plasmid DNA into E.Coli or yeast. JM 109 high efficiency competent E.Coli cells (Promega, Madison, USA) were used in this experiment for transformation. E. Coli is a rod shaped bacteria with a circular chromosome of about 3 million base pairs long.

Procedure

Polypropylene culture tubes were chilled on ice. Frozen competent cells were removed from -70°C, placed on ice for 5 minutes and thawed. The competent cells were mixed by flickering gently and 100 µl of it was transferred to each chilled culture tube (×2). Fifty nanogram of DNA containing either pcDNA3 with myosin enhancer, or pcDNA3 with myosin enhancer, and IGFBP-4 insert was added to competent cells. The tubes were flickered several times and kept on ice for 10 minutes.

Subsequently the cells were heat shocked for 45 seconds in a water bath at 42°C. Following this, the tubes were placed on ice for 2 minutes again. Nine hundred microlitres of cold SOC medium (at 4°C) was added to each tube and incubated for 60 minutes at 37°C in a shaking platform at 225 rpm. Glucose in the SOC medium enhances bacterial growth. The tubes were centrifuged at 1000 G for 10 minutes and pellets were resuspended in 200 µl of Luria - Bertani (LB) medium. The transformed cells were plated on LB plates containing 100 µg/ml of ampicillin, 0.5 mM of

isopropyl-beta-D-thiogalactopyranoside (IPTG) and 40 μg/ml of X-Gal and incubated overnight at 37°C. On the following day, blue colonies were picked and serially plated. The procedure is described in the next paragraph.

2.1.3 E.Coli culture

E.Coli containing pcDNA3 with and without IGFBP-4 inserts was grown at 37°C on LB agar plates with 100 μg/ml ampicillin overnight. A single colony of the bacteria was picked up from a freshly streaked selective plate and inoculated, to produce a start culture of 10 ml LB medium in a 30 mls universal container containing the ampicillin (50 μg/ml). It was incubated for 6 hours at 37°C with vigorous shaking (225 rpm). 1 ml of the start culture was added to 100 mls of LB media, which was prepared in sterile conical flasks and incubated overnight in a rotating platform at 225 rotations per minute at 37°C.

2.1.4 Plasmid extraction by Miniprep

Miniprep kit (Qiagen, Crawley, UK) was used for preliminary extraction of plasmids from E.Coli. One ml of LB medium containing overnight cultures of E.Coli was centrifuged in appendarf tubes at 13,000 rpm for 5 minutes. The bacterial pellets were resuspended in 250 μl of buffer P1, and 250 μl of buffer P2 was added, and the tubes were gently inverted 6 times to mix the contents. Then 350 μl of buffer N3 was added and the tubes were inverted 6 times to avoid any precipitation. The tubes were centrifuged for 10 minutes and the supernatant solution was applied to QIA prep spin column in a 2 ml collection tube. The spin columns/collection tubes were centrifuged for 1 minute and flow through was discarded.

Spin columns were washed by adding 0.75 mls of buffer PE and centrifuging them for 60 seconds. The flow through was discarded and centrifuged again for another 1

minute to remove residual buffer. Then the DNA was recovered with sterile PBS. The plasmid DNA was stored at - 20°C.

2.1.5 Sequencing

The DNA was sequenced at MWG biotech, Ebersberg, Germany. For sequencing, 2 μg of plasmid DNA was suspended in 20 μl in phosphate buffered saline and air dried in a centrifuge for 30 minutes. Sequencing results were compared with standard DNA sequences that are available in PubMed. It confirmed the presence of mammalian expression vector and IGFBP-4 cDNA in the plasmid. The results are shown in the appendix. Once the sequencing was confirmed, the E.Coli cells were grown further, and plasmids were extracted in large quantities using Endo Free Maxi Plasmid Extract Kit (Qiagen, Crawley, UK).

2.1.6 Plasmid extraction and purification

Pre-procedure preparations

Plasmid DNA isolation and purification was performed using the Endo Free Plasmid Maxi Kit (Qiagen, Crawley, UK). Before starting the purification of the plasmid, RNase A solution was added to Buffer P1. One vial of RNase A was used (spinned down briefly before use) per bottle of Buffer P1, to give a final concentration of 100 pg/ml. To prepare endotoxin-free 70% ethanol, 40 ml of 96 -100% ethanol was added to the endotoxin-free water supplied with the kit. Buffer P3 was pre-chilled to 4°C.

Alkaline lysis of bacterial pellet

To begin with, the bacterial cells were isolated by centrifugation at 6000G for 15 minutes at 4°C. 6000G corresponds to 6000 rpm in Sorvall GSA rotors. All traces of

supernatants were removed by inverting the open centrifuge tube until all medium had been drained. The bacterial pellet was resuspended in 10 ml of Buffer P1 (to which RNase was already added) completely by vortexing or pipetting up and down until no cell clumps remain. 10 ml of Buffer P2 was added, mixed gently but thoroughly by inverting 4-6 times, and incubated at room temperature for 5 minutes. After use, the bottle containing Buffer P2 was closed immediately to avoid acidification from CO_2 in the air. During the incubation the QIAfilter Cartridge was prepared. The cap was screwed onto the outlet nozzle of the QlAfilter Maxi Cartridge.

Neutralisation and precipitation genomic DNA

10 ml of chilled Buffer P3 was added to the lysate and mixed gently immediately by inverting 4-6 times. The lysate was poured into the barrel of the QIAfilter Cartridge immediately and incubated at room temperature for 10 minutes.

Removal of precipitates and endotoxins

The cap was removed from the QlAfilter Cartridge outlet nozzle and the plunger was gently inserted into the cartridge and the cell lysate was filtered into a 50 ml tube. Approximately 25 ml of the lysate was generally recovered after filtration. Then 2.5 ml of Buffer ER was added to the filtered lysate, mixed by inverting the tube approximately 10 times, and incubated on ice for 30 min.

Adsorption of plasmid DNA and washing

While the lysate was incubating, Qiagen-tip 500 was equilibrated by applying 10 ml of Buffer QBT, and allowed to empty by gravity flow. Flow of buffer began automatically by reduction in surface tension due to the presence of detergent in the equilibration buffer. Then the filtered lysate was applied to the Qiagen-tip and

allowed to enter the resin by gravity flow. Once the column was empty, the Qiagen-tip was washed with 2 x 30 ml Buffer QC by gravity flow.

Elution of plasmid DNA

Plasmid DNA was eluted with 15 ml of Buffer QN and collected in a 30 ml pyrogen-free glass tube, and the DNA was precipitated by adding 10.5 ml (0.7 volumes) room temperature isopropanol (VWR international, Lutterworth, UK). It was mixed thoroughly and centrifuged immediately at 11,000 rpm for 30 minutes at 4°C in a Sorvall SS-34 rotor. The supernatant was carefully pipetted out which left the DNA pellet at the bottom of the glass tube.

Purification of the plasmid DNA

The DNA pellet was washed with 5 ml of endotoxin free room temperature 70% ethanol and centrifuged at 9000 rpm for 10 min. The supernatant was pipetted without disturbing the pellet. The pellet was air-dried for 5 minutes and the DNA was redissolved in a suitable volume of sterile endotoxin free PBS. To determine the yield, DNA concentration was determined by UV spectrophotometer and the inserts were analysed on an agarose gel by electrophoresis.

Quantification of the plasmid DNA

The plasmid was quantified in a Gene spec I Spectrophotometer. After opening the software, PBS was used as baseline and an UV spectrometry reading was obtained. Then the 1 μL cuvette was washed with deionised water and ethanol, and plasmid was quantified. When the quantification graph (figure 2.3) was not smooth, the plasmid was diluted and measured again. In between the measurements, the cuvette was cleaned with deionised water and alcohol.

Storage of the transformed E.Coli

One ml glycerol was added to 9 ml of LB media and autoclaved at 121°C for 15 minutes which formed 2x freezing medium. Half ml of culture (transformed E.Coli) from the flask was added to 0.5 ml of this freezing medium and mixed well in a 1.5 ml tube which was stored at –70°C.

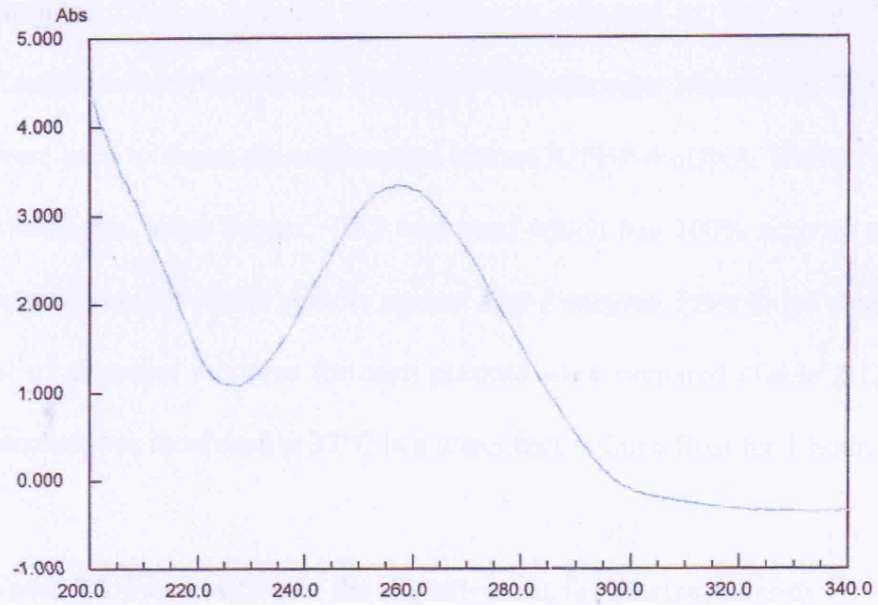


Figure 2.3 showing graph of plasmid DNA which was quantified using gene spec.

Result

230.0 (nm)	260.0 (nm)	280.0 (nm)	Ratio	Conc (ug/uL)	Pure (%)	Flag
1.167	3.293	1.541	2.137	1.65	118.7	H

Date : 04/3/2 15:52
Sample : Vector
Comment :

Instrument
Integration : 32

DNA Parameter
Mode : dsDNA
Dilution : 10.00
Background : Off
Factor : 50.0
Pure : ON
P.Conc : Off
M.Conc : Off
Unit : ug/uL

Upper : 100.0
Lower : 0.0
Expeted Value : 1.800

Figure 2.4 showing a typical measurement of DNA using gene spec

Confirmation of IGFBP-4 insert by agar gel electrophoresis

To confirm the presence of IGFBP-4 insert, plasmid DNA was cut with restriction endonucleases and gel electrophoresis was performed

Digestion of plasmid DNA with restriction endonucleases

Restriction endonucleases recognise short DNA sequences and cleave the double stranded DNA at specific sites within, or adjacent to, the recognition sequences. Restriction enzymes *Eco* R I and *Kpn* I (Boehringer Mannheim, West Sussex, UK) were used to digest the sequence to extract IGFBP-4 cDNA. Buffer 'A' (Boehringer Mannheim, West Sussex, UK) was used which has 100% activity against *Eco* R1 enzyme, and 75-100% activity against *Kpn 1* enzyme. Prior to gel electrophoresis, 20 µl of digestive mixtures for each plasmid were prepared (Table 2.1). The reaction mixture was incubated at 37°C in a water bath using a float for 1 hour.

Table 2.1 Composition of the digestive mix for electrophoresis

Component	Quantity (µl)
DNA	2
Enzyme Kpn-1	1
Enzyme ECOR-1	1
Buffer A(X10)	2
BSA	0.5
Water	13.5
Total	20

Preparing the gel

One gram of electrophoresis grade agar was added to 100 ml of 1% TAE buffer and heated in a microwave oven for 1.45 minutes to dissolve it. One µl of ethadium bromide (Invitrogen, Paisley, UK) was added to 25 ml of gel. The ethadium bromide allowed easy visualisation of the DNA under UV light so that it could be

photographed after electrophoresis. It was cooled to 55°C and poured onto a gel platform with a 12 lane gel comb to generate wells.

Loading and running the gel

Once the gel was set, the comb was removed. Sufficient electrophoresis buffer was added to the gel tank to cover the gel to a depth of about 1 mm. DNA samples were prepared by adding loading buffer (6×, Fermentas, York, UK). Samples were loaded into the wells with a pipette. One kb DNA ladder (Fermentas, York, UK) was used to compare the results of electrophoresis. Electrophoresis cables were attached and voltage was set at 80 Volt for 40 minutes (1-10 Volts/Cm of the gel). The DNA ran towards the positive (red) electrode. The power supply was turned off once the Bromophenol blue dye solution from the loading buffer had migrated to a distance sufficient for the separation of the DNA fragments.

Visualising the DNA

After electrophoresis the gel was taken to a UV spectrophotometer (Bio-rad Gel doc 2000) where the bands could be visualised and printed. The bands were compared with a DNA ladder, and sizes of the DNA bands were identified.

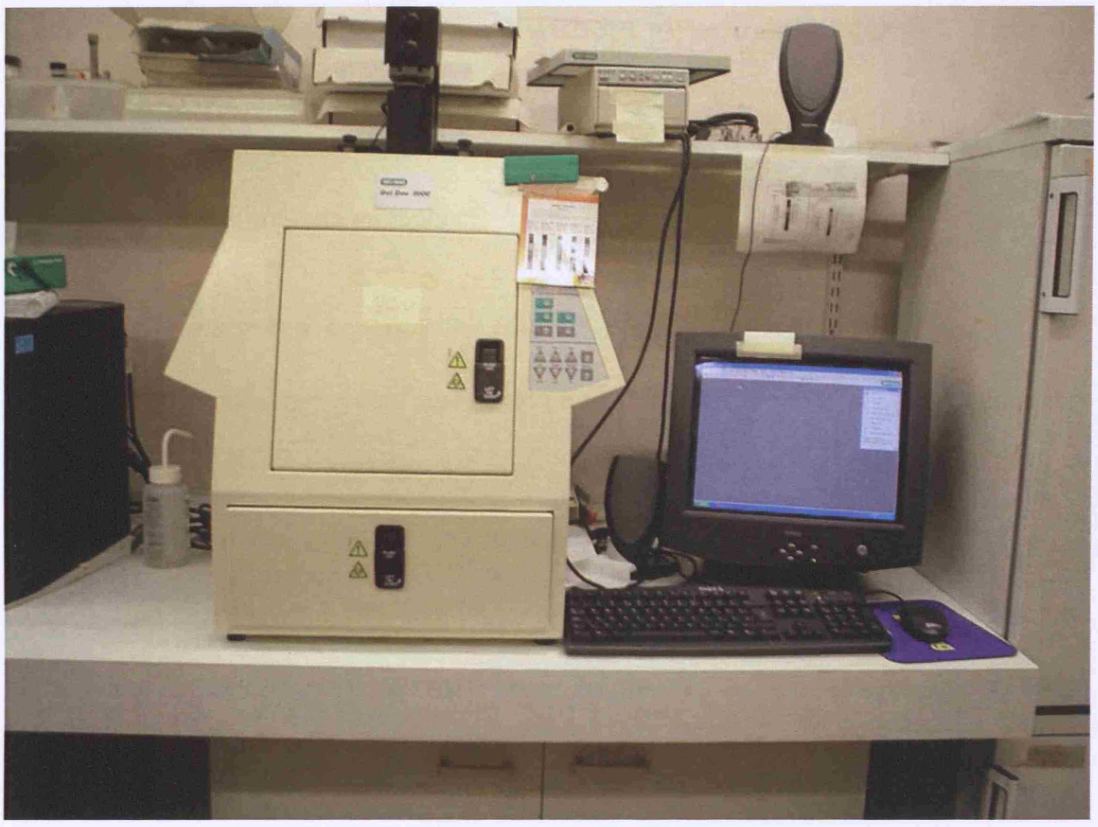


Figure 2.5 Bio-rad Gel doc 2000 for visualising DNA bands in gel. The gel is kept inside the white cabinet (shown in the middle of the picture) and DNA is visualised with ultraviolet rays and will appear on the computer screen.

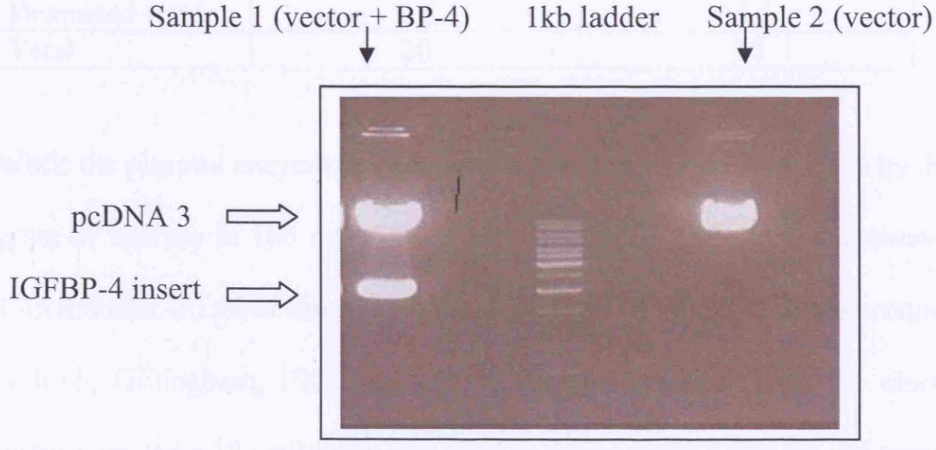


Figure 2.6 showing visualisation of DNA with UV light. IGFBP-4 insert is clearly seen.

The plasmid samples were sent to MWG, Ebersberg, Germany, for confirming sequence again, prior to establishing the early gene transfer model.

2.1.7 Preparation of gene construct without myosin enhancer

The aim was to produce a plasmid containing vector DNA and IGFBP-4 DNA insert without light chain myosin enhancer.

1. ***Gel electrophoresis to separate the IGFBP-4 insert and vector DNA***

The plasmid containing myosin light chain and IGFBP-4 insert was quantified using Genespec1. The vector pcDNA 3.1 was a given as a gift by Mr David Sutton, Molecular Tissue Repair Unit, The Royal Free and University College Medical School, London. Plasmid DNA and a restriction enzymes mixture was prepared as shown in table 2.2 and incubated for 2 hours 30 minutes at 37°C water bath.

Table 2.2 showing DNA enzyme mixture for electrophoresis

Component	IGFBP-4 mix (µl)	Vector mix (µl)
DNA(plasmid)	10	2
EcoR1	1	1
Kpn1	1	1
BSA	0.5	0.5
Buffer A	2	2
Deionised water	5.5	13.5
Total	20	20

While the plasmid enzyme mixture was incubating, 1% gel was made by dissolving 1 gram of agarose in 100 ml of TAE (1×) and heating this in a microwave oven for 1.45 minutes. 25 ml of this solution was taken and 1 µl of ethadium bromide (Sigma-Aldrich, Gillingham, UK) was added. This was poured in to the electrophoresis instrument and a 10 well comb was used to create wells. Once the gel was set, 1 µl of loading dye (6×) (Fermentas, York, UK) was added to each DNA-Enzyme mix and poured into different wells. One kb DNA ladder (Fermentas, York, UK) was used as a ruler. The gel was run for 40-60 minutes at 104 volts.

2. *Extraction of DNA from gel*

The areas of gel containing IGFBP-4 insert and vector were excised under UV light guidance in a dark room and kept in two different appendarf tubes. DNA was extracted by QIAEX – II Agarose Gel extraction method (Qiagen, Crawley, USA).

Steps of DNA extraction from gel

Gel was weighed and 3 times volumes of Buffer QX1 to 1 volume of gel was added and vortexed. 30µl of buffer QIAEX-II solution was added to each and incubated at 50°C water bath for 10 minutes and vortexed every 2 minutes. They were centrifuged for 30 seconds and supernatant removed, and the pellet was washed with 500 µl of buffer QX1. The pellet was washed twice with 500 µl of buffer PE (70% alcohol) and air dried in vacuum pump for 10 minutes to produce white DNA. 20µl of nuclease free, bacteria free, water was added to each. The pellet was vortexed to resuspend it. It was centrifuged and supernatant was stored in a clean sterile tube. A small amount of DNA was run in the gel to confirm that the extraction had DNA.

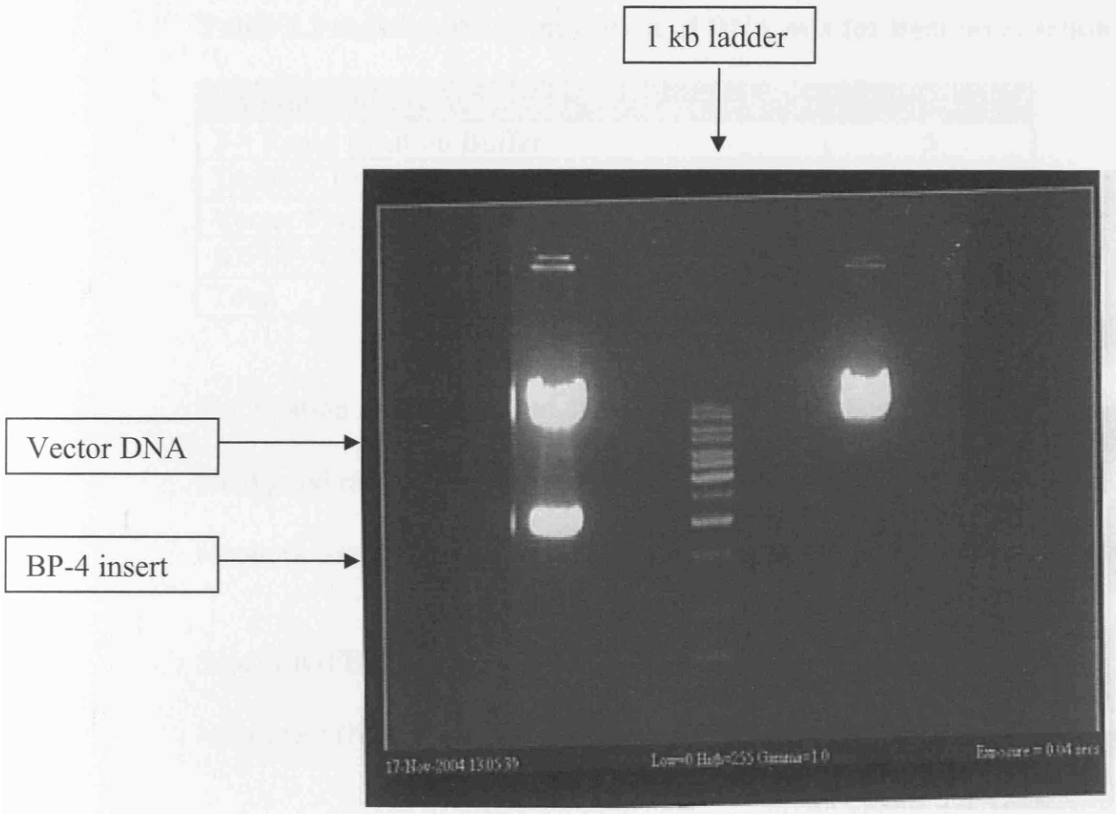


Figure 2.7 showing gel electrophoresis of plasmids which were digested with restrictive endonucleases. The IGFBP-4 insert is obvious.

Purification of DNA extracted from the gel

Ten microlitres of the DNA was made up to 100 µl by adding deionised water. To this 100 µl, 10 µl (10%) of 3 mMol sodium acetate and 2.5 times (250 µl) of 100% methanol were added and incubated overnight at 4°C.

Ligation of DNA

PGEM - T Easy Vector System II kit (Promega, Madison, USA) was used for ligation. On the first attempt the DNA mix was prepared as shown in Table 2.3.

Table 2.3 showing the composition of DNA mix for ligation reaction

Component	Quantity (µl)
2 × Rapid Ligation Buffer	5
T4 DNA Ligase	1
Vector Plasmid DNA	2
IGFBP-4 DNA	2
Total	10

The ligation mix was kept at 4°C in a cold room overnight. This had twice failed to yield good results. So ligation was repeated according to the manufacturer's recommendations, as below (Table 2.4).

Size of IGFBP-4 insert = 2.2 kb Size of vector = 8kb

$$\text{Nanogram (Ng) of insert required} = \frac{\text{Ng of Vector} \times \text{kb insert} \times \text{ratio}}{\text{Kb vector}}$$

$$\text{Ng of insert required} = \frac{50 \text{ ng vector} \times 2.2 \times 3}{8}$$

$$= 41 \text{ Nanograms}$$

On measuring with Gene spec we found that both vector and insert were

0.07 µg/µl= 70 ng/µl

Table 2.4 showing composition of ligation mix

Component	Quantity (µl)
2 × Rapid Ligation Buffer	5.00
T4 DNA Ligase	1.00
Vector Plasmid DNA	0.71
IGFBP-4 DNA	0.59
Water	2.70
Total	10.00

After overnight incubation at 4°C, the competent cells (Easy Vector, Promega, Madison, USA) were transfected. LB agar plates were prepared. The following solution was used for each plate before plating the transfected cells.

Ampicillin- 40 µl of 50 mg/ml

IPTG - 40 µl of 100 mM

X-gal – 20 µl of 40 mg/ml

Total = 100 µl per plate

Transfection procedure

This transfection failed twice for no obvious reasons, so transfection was repeated with SURE (Stop Unwanted Rearrangement Events) (Stratagene, La Jolla, California, USA) competent cells.

SURE transformation protocol

At first, two appendarf tubes were pre-chilled on ice. Then competent cells were thawed on ice, gently flickered to mix and 100 µl of cells aliquoted to each of the pre-chilled tubes. 1.7 µl of β-mercaptoethanol was added to each tube and incubated on ice for 10 minutes, and the contents were swirled every 2 minutes. Then 2 µl of experimental DNA was added to one aliquot and 1 µl of the pUC 18 control DNA to the other aliquot, the tubes were swirled gently and incubated on ice for 30 minutes. To facilitate the entry of DNA into the cells, they were heat pulsed in a water bath at 42°C for 45 to 50 seconds and incubated on ice for 2 minutes. Then 0.9 ml of preheated warm LB medium was added, and incubated in a shaking platform at 37°C at 225 rpm.

Then 200 µl transformation mixture was plated on to LB plates containing Ampicillin, IPTG (Iso-propyl-1-thio-β-D-galactopyranoside) and X-gal (5-bromo-4-chloro-3-inodyl-β-D-galactopyranoside). These reagents provide blue-white screening. The plates were incubated overnight at 37°C. Transformation of vector

failed four times. Finally, the gel extraction was repeated and pcDNA 3.1 plasmid was extracted. The gel electrophoresis showed the presence of BP-4 insert (figures 2.8 and 2.9). Sequencing was performed in two places (Imperial College, London and MWG, Ebersberg, Germany). Neither of them confirmed the right sequence for the IGFBP-4.

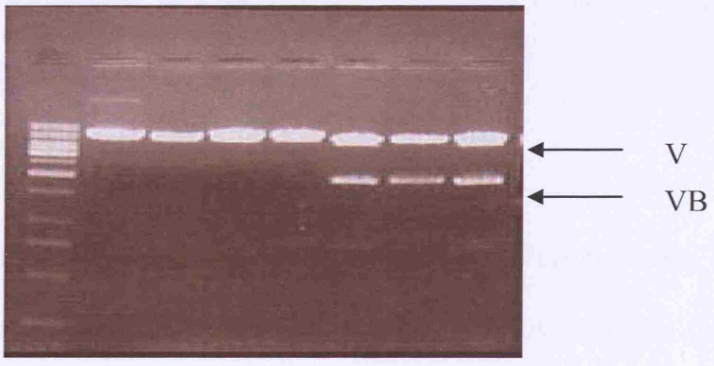


Figure 2.8 Gel electrophoresis showing plasmid of vector (V) and vector with IGFBP-4 insert without myosin enhancer (VB) , after cutting the plasmids with *Kpn* I and *Eco*R I restriction endonucleases (Miniprep).

Figure 2.9 Gel electrophoresis showing plasmids of vector with IGFBP-4 insert without myosin enhancer, after cutting the plasmids with Kpn I and EcoR1 restriction endonucleases (Maxi prep). (Key: V- plasmid vector, VB- plasmid with BP-4 construct)

2.2 Cell culture

2.2.1 Colon cancer cell line

HT-29 is a widely studied human colon adenocarcinoma cell line. It was originally isolated from a primary tumour in a 44 year old Caucasian female. On culture it forms a well-differentiated adenocarcinoma.

2.2.2 Culture

HT-29 cells (European Collection of Animal Cell Cultures, Porton Down, Dorset, UK) were cultured in 75 cm^3 flasks containing McCoy's 5A Medium (GIBCO, Paisley, UK) enriched with glutamine (2mM), 10% foetal bovine serum and 1% Penicillin (5000 unit/ml), and streptomycin (5000 μg/ml) at 37°C in an atmosphere of 5% CO_2. After 48 hours, the medium was extracted, the cells were washed twice in 10 mls of PBS, and the medium was replaced with 20 mls of fresh medium. Cells were cultured under standard conditions. The plastic wares were obtained from Marathan Ltd, London, UK.

2.2.3 Quantification and viability assessment

Prior to injection, cells were trypsinised, counted on haemocytometer and viability was assessed with Tryphan blue dye exclusion. Only cells with >90% viability were used for induction of cancer *in vivo*.

2.2.4 Passaging of cells

When the cells were 90% confluent they were passaged. The medium was extracted and the cells were washed twice in 10 mls of PBS. 2 mls of 0.25 % Trypsin-EDTA

(Invitrogen, Carlsbad, CA, USA) was added to the flask and the cells were incubated at 37°C for 5 minutes. The flask was then shaken to release the cells, and it was confirmed with microscope. Then 5 mls of fresh medium containing serum was added to neutralise the trypsin. The cells were centrifuged at 900G for 5 minutes. The supernatant medium was discarded, and the pellets of cells were resuspended in 10 mls of fresh medium. Two millilitres of the cell suspension was placed in each of 75 cm^3 flask and 18 mls of McCoy's medium, enriched with foetal calf serum and antibiotics, was added.

2.2.5 Preparing for animal injection

Five flasks of 90% confluent cells were chosen and washed with 10 mls of PBS twice. Then they were trypsinised, neutralised and centrifuged at 900G for 5 minutes. The cells were resuspended in 10 mls of PBS (GIBCO, Paisley, UK). Cell counting was performed using a haemocytometer, which showed a counting of $500x10^4$ cells. The counting is accurate only between 30 and 300×10^4, so the sample was rediluted and recounted. 100 µl of the sample was taken and it was added to 100 µl of Tryphan Blue. Dead and live cells were counted. A final concentration of 12 mls of cells at 5×10^6/ml of more than 95% viability was chosen, and 0.6 mls (3×10^6/ml cells in PBS) was injected subcutaneously into the flank of each nude mouse. In the early gene transfer model, 10 flasks full of cells were used at the same concentration.

2.3 Animal model

The studies were conducted under a project licence (PPL70/5232, 15 October 2000) granted by the Home Office in accordance with the Animals (Scientific Procedures) Act 1986. A personal licence (PIL70/18595) was obtained from the Home Office, UK to carry out regulated procedures on living animals according to the Animals (Scientific procedures) Act 1986.

2.3.1 Animals

In the late gene transfer model, 13 male athymic MF1 nu/nu nude mice of 4-6 weeks age, each weighing between 25 - 30 grams, were used. The mice were bought from the Comparative Biology Unit, University College London, Hampstead campus, London. In the early gene transfer model 18 male nude mice were used. The particular strain lacked functioning T cells (cell mediated immunity) because it had no thymus, and there was no host/graft disease that could reject the tumour cells. Four animals were kept in each cage. The cages were clearly marked and animals were identified by making holes in their pinna with an ear punch.

The animals were grown and maintained under specific pathogen free conditions, using a laminar airflow rack, and had free access to sterilised food and autoclaved water. The cages were individually ventilated and the animals were provided with sterilized bedding. There were 12 hourly alternate light dark cycles, and only those wearing a gown and gloves were allowed into the room (restricted entry).

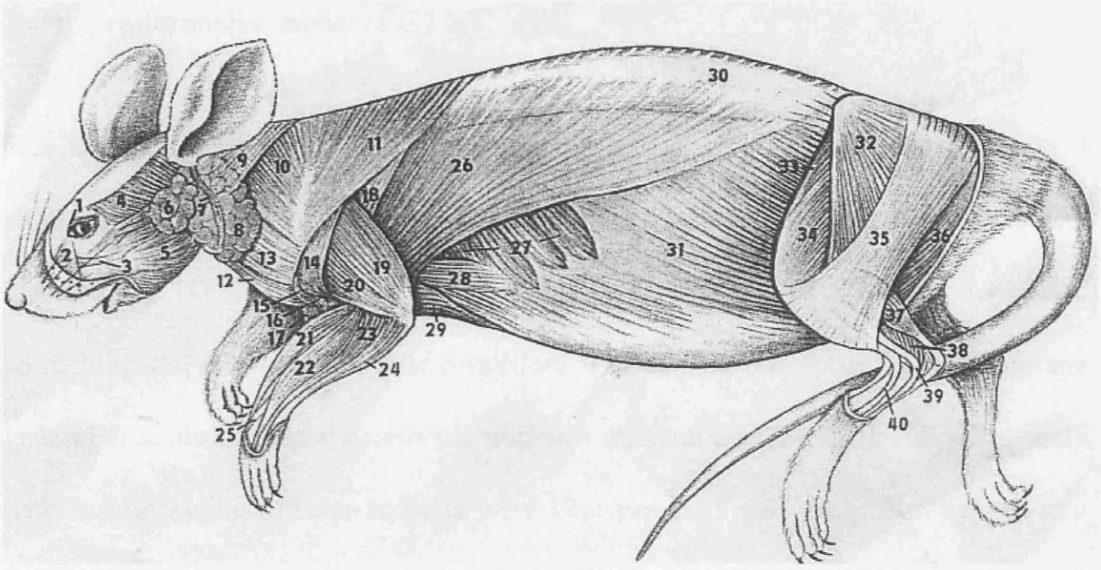


Figure 2.10 Subcutaneous muscles of a mouse. It shows the subcutaneous plane of a mouse which has numerous skeletal muscles. (Reproduced from 'A Colour Atlas of Anatomy of Small Laboratory Animals; Volume 2: Rat. Mouse. Hamster. Authors P.Popesko, V.Rajtova, J Horak, year 2003, published by Wolfe)

2.3.2 Tumour induction

Late gene transfer model (LGTM)

Using disposable insulin syringes, nude mice were subcutaneously inoculated with 0.6 ml of 5×10^6 HT-29 cells per mouse in PBS, under light isofluorane anaesthesia. The animals were observed under Home Office (UK) regulations. On day 8, 12 animals showed palpable and visible tumours. The animals were divided into two groups (n=6), and they received either 150 μg of mammalian expression vector containing IGFBP-4 cDNA, or vector alone, in PBS by peritumoural injection. The thirteenth animal also developed a tumour at the middle of the second week. The animals were sacrificed on day 25.

Early gene transfer model (EGTM)

The animals were randomly divided into 3 groups of 6 animals each. Group 1 received just HT-29 cells alone in PBS, group 2 received a mixture of control plasmid and HT-29 cells, and group 3 received a mixture of HT-29 and plasmid containing IGFBP-4 cDNA. The procedure was carried out under light enfluorane general anaesthesia, and the cells/plasmid was injected into the flank subcutaneously with insulin syringes. Four animals were kept per cage and each cage was clearly marked. Animals in the cages were identified by ear punch. The animals were observed for their well being. The tumour size was measured at two time points. The experiment was terminated 4 weeks after tumour induction.

Figure 2.11 Subcutaneously induced tumours in a MF1 nude mouse after inoculation with HT-29 cells, and the photograph was taken 2 ½ weeks after plasmid therapy. The tumour is seen as a pink lump.

2.3.3 Observation and tumour measurements

During the entire experimental period the animals were observed for their wellbeing. The observations include daily assessment for their activity, food and water intake,

and their response to noise. The tumour sizes were measured in two dimensions with a digital vernier calliper, and the animals were weighed on a weekly basis.

2.3.4 Necropsy and tumour sampling

The experiment was terminated when the animals were moribund, or the tumour was >10% of body weight, and tumour tissues were harvested. The mice were killed by Schedule 1 method (cervical dislocation) and the tumours were dissected out, weighed, their sizes were measured and then divided into several portions for subsequent examination.

Tumour harvesting

Prior to tumour harvesting, cryovials, small universal containers and cork for frozen sections were clearly marked for identification. 10% formalin, liquid nitrogen, isopentane and optimum cutting temperature compound (OCT) (VWR Ltd, Lutterworth, UK) were made available for tumour sampling.

Sample for histopathology

The tumours were cut longitudinally and one portion was fixed in 10% formalin for paraffin section.

Sample for frozen section

Frozen samples were prepared for immunostaining and TUNEL assay, by embedding the tissue in OCT compound and rapidly freezing them in pre-cooled isopentane (BDH, Lutterworth, UK). The frozen samples were stored at - 70°C.

Sample for Western blot

Uncrushed and snap frozen tumour samples were collected in special cryovials for Western blot, cooled in liquid nitrogen and stored at - 70°C.

Serum

After sacrificing the animal by cervical dislocation, blood was collected by cardiac puncture with a 22 G needle attached to 5 ml syringe. The sample was centrifuged, the serum was separated and stored at -70°C. It was not possible to collect blood samples from all the animals (too little) and they hemolysed easily.

Muscle sample and sample for electron microscopy

Samples from skeletal muscle adjacent to the tumour were obtained for immunostaining. The tumour tissues were divided and ~1 mm^3 samples, one from each of the quadrants of cancer tissue, were obtained and stored at 4°C in gluteraldehyde based solution for transmission electron microscopy.

2.4 Tumour histology

2.4.1 Macroscopy

The tumours were examined for any local invasion to skin and muscle, areas of haemorrhage or necrosis as well as local spread.

2.4.2 Haematoxylin and eosin (H&E) staining

The H&E staining was performed by Ms Maroc of the Pathology Department at the Hammersmith Hospital. After embedding the tissues in paraffin, consecutive 5 μm sections were cut and mounted on polysine slides (VWR International Ltd, Lutterworth, UK).

The sections were deparaffinised with 2 changes of xylene, 10 minutes each. This was followed by rehydration in 2 changes of absolute alcohol 5 minutes each, 95% alcohol for 2 minutes and 70% alcohol for 2 minutes. This procedure removed the xylene. Then the sections were washed briefly in distilled water to remove the alcohol. Nucleus was stained by incubating with Harris haematoxylin solution for 8

minutes. Then the sections were washed in running tap water for 5 minutes. This was followed by incubating the sections in 1% acid alcohol for 30 seconds. The sections were washed with running tap water for 1 minute again, then blued in 0.2% ammonia water for 30 seconds to 1 minute, and washed in running tap water for 5 minutes. The slides were then rinsed in 95% alcohol, 10 dips.

The sections were counterstained with eosin Y solution for 30 seconds to 1 minute, and dehydrated through 95% alcohol, 2 changes of absolute alcohol, 5 minutes each, then washed in 2 changes of xylene, 5 minutes each. Finally the slides were mounted with xylene based mounting medium. After staining with H&E, the tumour sections were assessed for cellular pleomorphism, gland formation, mucous production and necrosis.

2.4.3 Cell death scoring

The degree of cell death of the tumours was scored according to the method described previously by Takei et al [Sawaoka et al., 1998;Takei et al., 1990]. Necrosis found in the <1 %, 1-20 %, 21-40%, 41-60%, 61-80% and 81-100% of the region of interest was scored as 0, 1, 2, 3, 4 and 5, respectively [Sawaoka et al., 1998;Takei et al., 1990].

2.4.4 Mitosis counting

During cellular proliferation, instead of nucleus, the chromosomes are visible as tangled, dark-staining threads. These are known as mitotic figures. If nuclear membrane is seen, it is not a mitotic figure. Mitosis (figure 2.12) reflects proliferation of tumour cells. So the mitotic figures were counted blindly on 10 random high power fields (× 400) on each section which were stained with H&E

[Mills et al., 2001]. Mitotic figures in anaphase through early telophase were included in the counting.

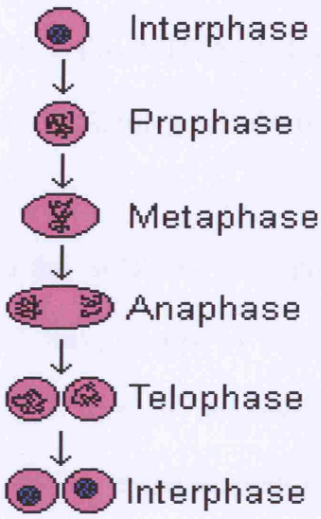


Figure 2.12 showing the stages of mitotic division which occurs in all mammalian cells except reproductive cells. It is a form of asexual cell division. The numbers of cells in mitosis is a measure of cellular proliferation.

2.5 Apoptosis assessment

2.5.1 TUNEL assay

Terminal deoxynucleotidyl transferase biotin-dUTP Nick End Labelling (TUNEL) assay was performed using Apotag-red kit (Serologicals Corporation, Temecula, CA, USA).

At the beginning of the experiment a few solutions were prepared. They included phosphate buffered saline (PBS), which was prepared by dissolving 5 tablets of PBS (Sigma-Aldrich, Gillingham, UK) in 1 litre of deionised water, 5% formalin (prepared by diluting 10% formalin with an equal amount of PBS), ethanol-acetic acid mixture (prepared by adding 40 ml of ethanol to 20 ml of acetic acid under hood), enzyme mixture (prepared by adding 42 µl of reaction buffer to 18 µl of TdT per slide) and stop wash buffer (prepared by adding 1 ml of stop wash buffer to 34 ml of deionised water in a coplin jar). Finally, labelling rhodamine mix was prepared (68 µl of blocking solution to be added to 62 µl of anti-digoxigenin rhodamine), mixed well and protected from light.

Method

Tumour sections were fixed in 5% formalin for 10 minutes at room temperature. Then they were washed in PBS twice, each wash for 5 minutes. This was followed by immersing them in pre-cooled ethanol: acetic acid mixture (2:1) for 5 minutes at -20°C in a coplin jar. The slides were not allowed to dry as this solvent permealises the cells. Then the slides were washed with PBS (2 changes of 5 minutes each) to remove these chemicals.

Next step was application of undiluted equilibration buffer directly to the specimen, and to incubate them for 10 minutes. The crucial step being application of TdT

enzymes mixture and incubation in a humidified chamber for 1 hour at 37°C. Then the specimens were immersed in colpin jars containing stop wash buffer, agitated for 15 seconds and incubated for 10 minutes at room temperature. The slides were washed with PBS (3 changes of 1 minute each), and anti-digoxigenin mixture (rhodamine) was applied and incubated in a humidified chamber at room temperature for 30 minutes. Exposure to light was avoided. Addition of DAPI in 1/1000 dilution was optional.

Finally, the slides were washed with PBS (4 changes of 2 minutes etc), protected from light and mounted using AF-1 (Citiflour, Leicester, UK) mounting media. Nail polish was used to fix the cover slip. The apoptosis was evaluated with a confocal microscope (Nikon, Kanagawa, Japan).

Figure 2.13 showing the Leica CM 3050 cutting machine that was used to cut the tissue section from frozen samples. The samples were fixed to a mount and cut to desired thickness at the temperature of -20°C.

Using the machine shown above (figure 2.13) 10 μm thick tumour sections were cut from frozen samples. Immediately after cutting, the sections were applied on polysine slides and stored at - 70°C, and used for all frozen immunostaining, including TUNEL assay. The slides were clearly marked with pencil/pen for identification, and two or three sections were applied per slide.

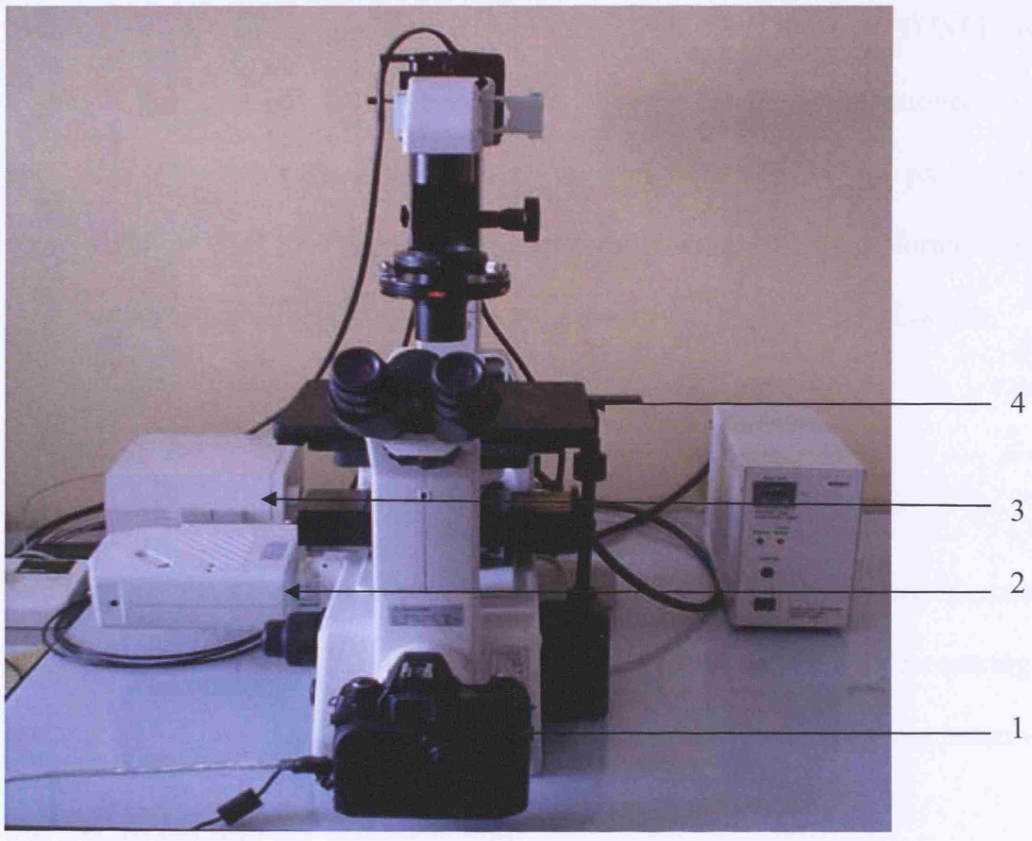


Figure 2.14 showing the Nikon con-focal/fluorescent microscope that was used to visualize tumour sections. (*Key* 1- Digital camera, 2 – Fluorescence lamp, 3 – Scanner camera, 4 – Platform for slides)

2.5.2 Quantification of the results

Apoptotic cells were identified by red fluorescence, and the counting was carried out blindly by two observers. For quantification of apoptosis, 10 random fields were

chosen for each sample at × 200 magnification [Bruns et al., 2000], and the average cell count was used. The apoptotic index was calculated using the formula as described previously [Sawaoka et al., 1998] (Apoptotic Index (%) = (apoptotic cells / total number of cells ×100) .

2.5.3 Electron microscopy

Transmission electron microscopy was performed to assess the ultra structure of cancer cells to confirm apoptosis and correlate them with those of TUNEL assay findings. The protocol for processing the tumour sample is mentioned in the appendix. Ultra thin tumour sections (60-90 nm) were viewed and photographed using a Philips CM120 transmission electron microscope. It was performed by Mr Innis Cladsworthy of Electron Microscopy at the Royal Free Hospital, London.

2.6 Assessment of Bcl-2 and Bax expression

Since the results of apoptotic index showed significant difference between control and BP-4 group tumours, the mechanism of apoptosis was assessed by measuring the Bax and Bcl -2 proteins. Western blot was used for quantification of these proteins.

2.6.1 Western blot for Bcl-2 protein

Total protein from the tumour samples were quantified by modified Lowry protein assay (Pierce Biotechnology, Rockford, IL, USA).

Protein extraction

The tissue samples which were stored at -70°C were obtained and powdered by crushing them with the help of liquid nitrogen. Then 7 ml of preparatory buffer mixture (Table 2.5) was prepared in the following combination.

Table 2.5 Composition of preparatory buffer for protein extraction

Contents	Volume (µl)
Reporter Lysis Buffer (Promega) Proteinase Inhibitor Phosphate Buffered Saline	1400 700 4900

Five hundred microlitres of the buffer was added to each tumour sample and vortexed for 15 minutes. Then the samples were centrifuged for 5 minutes, and the supernatant solution was aliquoted in previously marked appendarf tubes (3 for each sample). The tubes were stored at -70°C.

Modified Lowry protein assay

This assay was performed in two steps. The first step was the preparation of standard dilutions of albumin, and the second step was quantification. Standard dilutions of bovine serum albumin (BSA) solutions were prepared in varying concentrations as follows (Table 2.6). One ml ampoule containing 2 mg of BSA, that was part of the kit, was used in the preparation of standard albumin dilutions.

Table 2.6 Standard dilutions of BSA

BSA (µl)	PBS (µl)	Concentration (mg/µl)
0	200	0
0	200	0
8	192	0.08
16	184	0.16
24	176	0.24
32	168	0.32
40	160	0.40
80	120	0.80

Phenol reagent (Lowry kit) was mixed with an equal amount of de-ionised water (100 µl/sample). Tumour sample solutions were diluted 100 times (2 µl of sample to 198 µl of PBS).

Procedure for protein quantification

Two hundred microlitres of sample solutions and varying dilutions of BSA were taken in appendarf tubes. At 15-second intervals, 1 ml of modified Lowry reagent was added to tumour samples, mixed well and incubated for 10 minutes at room temperature. 100 µl of prepared IX Folin-Ciocalteu reagent (Lowry kit) was added and vortexed to mix the contents. 15 seconds intervals between the tubes were maintained. All the tubes were incubated at room temperature for 30 minutes. With the spectrophotometer set to 750 nm, the instrument was zeroed with a cuvette filled only with PBS. Then the average 750 nm absorbance values of the blank standard replicates was subtracted from the 750 nm absorbance values of all other individual standard, and unknown sample replicates. A standard curve of albumin was prepared by plotting the average blank-corrected 750 nm value for each BSA standard vs its concentration in µg/ml. Using the standard curve, the protein concentration of each unknown sample was determined.

Figure 2.15 showing the Shmadzu UV -160 A - UV visible reading spectrophotometer. Sample is kept in 1 µl cuvettes inside the reader, and total protein is quantified using ultra violet rays.

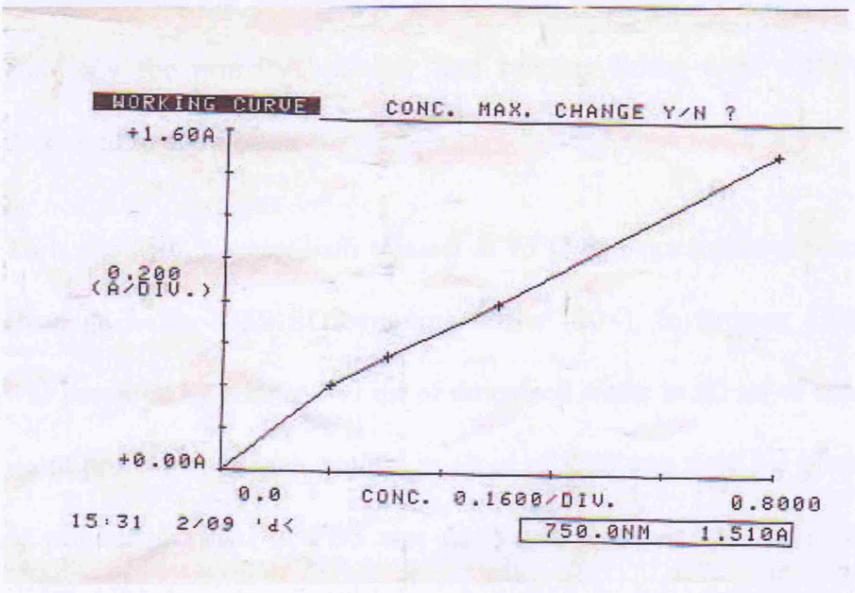


Figure 2.16 Standard curve that was prepared using albumin solution

Table 2.7 Quantitative analysis for total proteins in tumours (LGTM)

No	Absorption	Concentration (µg/µl)
B1	1.074	55
B2	0.839	42
B3	0.634	29
B4	0.565	25
B5	0.970	49
B6	1.035	53
M1	0.538	23
M2	0.479	19
M3	0.798	40
M4	0.538	23
M5	0.408	15
M6	0.366	14
P	0.640	29

(Key: B1 – 6 = IGFBP-4 plasmid treated, M1-6 = control plasmid treated, P = control – no plasmid given)

Western blot procedure

The procedure was the same for all Western blots that were performed in this project and only the primary antibody and running hours were different and they are described in appropriate places.

To begin with, a water bath was set at 95°C and run buffer-electrophoresis solution (Nupage buffer-MES SDS running buffer (20×), Invitrogen, Carlsbad, CA, USA) was prepared by adding 760 ml of deionised water to 40 ml of run buffer. About 50 µg of protein from each sample in 10 µl of PBS was used for blotting. Initially 1 µg of protein in 10 µl of PBS was used, but there was no detectable protein in the polyvinylidine fluoride (PVDF) membrane. So, throughout the project 50 µg of protein was used. To these 10-µl samples, an equal volume of sample buffer (Laemmli, 2×, Sigma-Aldrich Company Ltd, Gillingham, UK) was added and

thoroughly mixed. The samples were then denatured by heating them at 95°C for 5 minutes. Nupage - 12% Bis -Tris Gel (Invitrogen, Carlsbad, CA, USA) with 1.0 mm×10 wells or 15 wells was used for electrophoresis. Samples plus marker (See Blue Plus 2 Pre-stained Standard (1×) (Invitrogen, USA) was used as a ladder. The power source for electrophoresis was set at 150 voltage for 2 gels (75 each) and 40 mAmp for 2 gels (figure 2.17). The electrophoresis was run for 2 and 1/2 hours. Running time depended upon the size of the protein which needed to be isolated.

While the samples were running, transfer buffer, PVDF membrane, card boards and methanol were obtained. Nupage transfer buffer (20×) (Invitrogen, USA) 10 ml was made up to 200 ml with deionised water for 2 gels, thick blotting cards, 2 for each gel, and 1 cellulose membrane for each gel (Immuno-Blot PVDF membrane, Bio-rad, Hercules, CA, USA) were cut to the size of 8.5 × 7.5 cms and 20 ml of 100% methanol (VWR, Lutterworth, UK) was prepared.

Transfer of protein from the gel to the PVDF membrane was performed as follows. First the electrical power source was set at 25 volts (for any number of gel) and 75 amps for each gel for 45 minutes. The gel from electrophoresis was carefully isolated from the plastic case and immersed in the transfer buffer. The PVDF membrane was briefly immersed in methanol and it was followed by soaking them in transfer buffer. The transfer was done in a Bio-rad Transblot machine. PVDF membrane was applied on to a blotting card which was soaked in transfer buffer, and the gel was applied on the membrane. One or two blotting cards were applied on the gel and any trapped air in the sandwich was removed. Then electro blotting was performed. After transfer, the membrane was removed and soaked in ponceau solution to find out whether there

was any protein. This solution will stain all proteins. The membrane was then washed with plenty of deionised water to remove the ponceau solution. This is an optional step.

The membrane was immersed in 1% gluteraldehyde which was prepared by adding 1 ml of 50% solution (BDH, Lutterworth, UK) to 49 ml of deionised water. The membrane was incubated in 1% gluteraldehyde overnight in a cold room at 5°C.

The membrane was washed three times in deionised water, each wash for 5 minutes and blocked with freshly prepared 5% milk (Marvel semi-skimmed milk powder, 2.5 gms in 50 ml of PBS). This was followed by incubating the membrane with rabbit polyclonal anti Bcl-2 antibody (Santa-Cruz Biotechnology, Santa Cruz, CA, USA) at 1/200 dilution (10 ml of 5% milk to 50 µl of antibody) for 2 hours 30 minutes. This was followed by three washes with PBS, for 5 min each, to remove any excess of antibody.

The membrane was incubated with goat anti -rabbit antibody horse radish peroxidase conjugate (Dakocytomation, Carpinteria, CA, USA) at 1/2000 dilution (5 µl of antibody to 10 ml of 5% milk), for 45 minutes. The membrane was washed with PBS three times, for 5 min each, and Super Signal West Dura Extended Duration Substrate (Pierce, Rockford, IL, UK), which was freshly prepared (by mixing 1:1 solution peroxidase buffer to enhancer buffer) was applied and incubated for 5 minutes. The excess was drained and the membrane was covered with a plastic bag and X-rayed (Fuji films, Kanagawa, Japan) for varying durations.

Quantification of Western blot result

The X-ray film was scanned in a Bio-rad scanner, and the image was analysed with Bio-rad densitometry analysis software for Windows 2003. The density in relation to volume was calculated by the software. The results were plotted in Prism 4 software for statistical analysis.

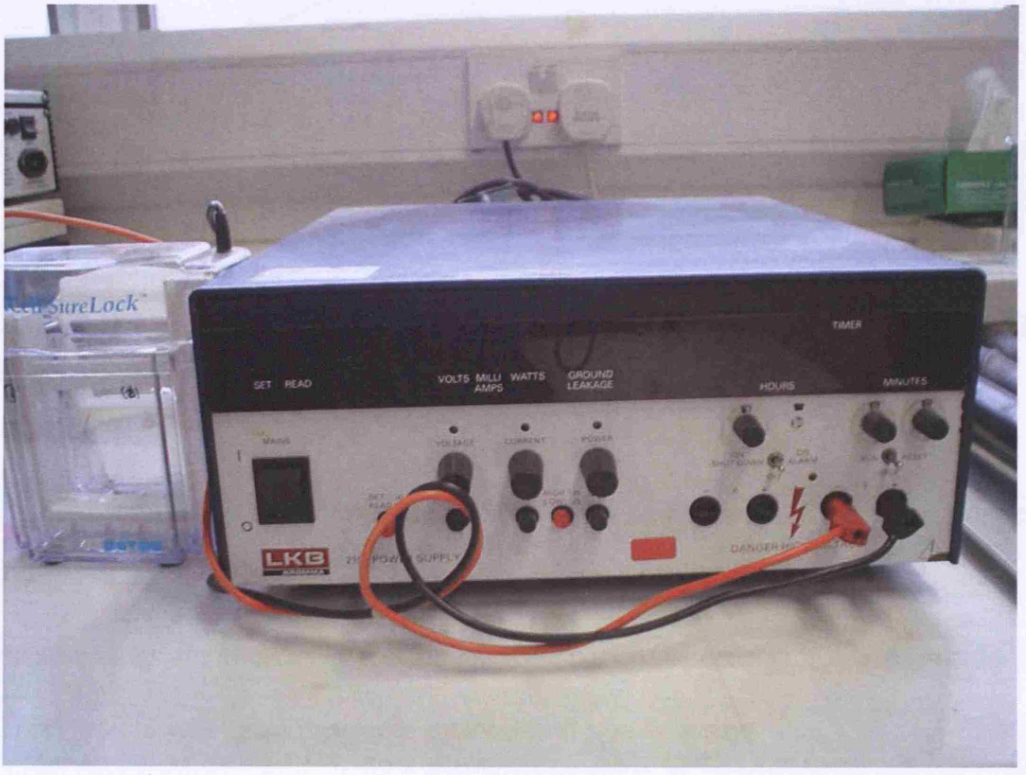


Figure 2.17 The power source and gel tank that were used for electrophoresis in Western blot. The gel is kept vertically in a plastic container containing run buffer (shown on the left). The instrument on the right provides the desired voltage and current for electrophoresis.

Western blot for the early gene transfer model

In the second (early gene transfer) model the procedure for Western blot was the same as for the first experiment. At first, a standard albumin curve was plotted (figure 2.18).

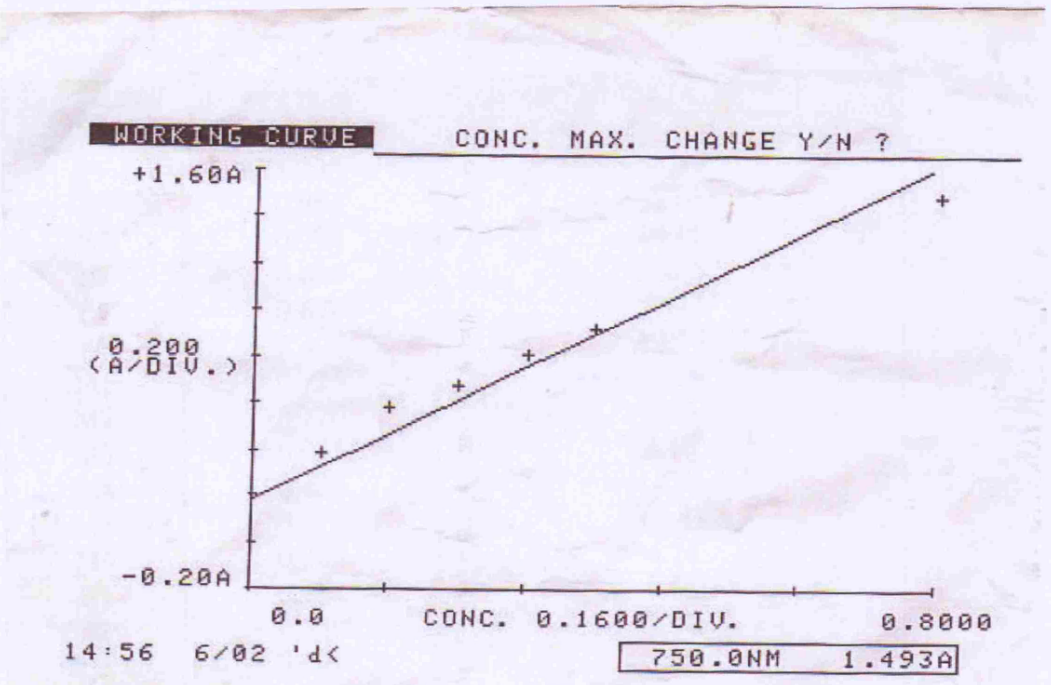


Figure 2.18 showing the standard albumin curve that was used for quantification of proteins from tumour samples in the second (early gene transfer) model.

Tumour samples were crushed into powder and total protein was extracted from each sample. After plotting the albumin curve, total protein from each tumour sample was measured, as for the first experiment, with an UV spectrophotometer (Table 2.8). Total protein was again measured by modified Lowry assay.

Table 2.8 Protein concentration in the tumour samples (EGTM)

Sample	Absorption	Concentration (μg/μl)
P1	1.233	58.49
P2	0.864	38.01
P3	0.794	34.11
P4	0.696	28.65
P5	0.674	27.44
P6	0.400	12.66
M1	0.725	30.29
M2	0.220	2.24
M3	0.245	3.63
M4	0.439	14.41
M5	0.410	12.81
M6	0.265	4.74
B1	0.264	4.66
B2	0.348	9.37
B3	0.249	3.84
B4	0.367	10.40
B5	0.342	9.03
B6	0.653	26.26

(Key: P1-6: control tumours without plasmid treatment

M1-6: tumours after control plasmid treatment

B1-6: tumours after BP-4 plasmid treatment)

2.6.2 Western blot for Bax protein

The method is the same as for Western blot used in Bcl-2, except that rabbit primary polyclonal Bax antibody (Santa Cruz, USA) was used in 1/200 dilution.

2.7 Assessment of the IGF system

The effect of the IGFBP-4 gene therapy on components of the IGF system was assessed. They included IGFBP-4, IGF-I, MGF (variant of IGF-I) and IGF-I receptor. Among these, IGF-I and MGF antibodies did not yield a good result, so the procedures for IGF-I and MGF staining were abandoned.

2.7.1 Western immunoblot for IGFBP-4

Here, primary rabbit anti-IGFBP-4 polyclonal antibody (Santa Cruz, USA) was used at 1/200 dilution. The remaining procedure was the same as for Bcl-2 described previously.

2.7.2 Immunostaining for IGFBP-4

Frozen tumour samples were cut into 10 μm thickness sections and 3 sections were applied per polysine slide (VWR International Ltd, Lutterworth, UK). They were marked and kept frozen at -70°C.

PBS was prepared by dissolving 5 tablets of PBS (Sigma-Aldrich, Gillingham, UK) in one litre of de-ionised water. The tumour sections were fixed with 5% formalin (10% formalin diluted with an equal amount of PBS) at 4°C for 10 minutes. The slides were washed with PBS containing 0.1% Bovine Serum Albumin (Sigma-Aldrich, Gillingham, UK) (prepared by adding 1 gram of BSA to 1 litre of PBS) - three times, each for 10 minutes. Then, circles were made around the sections with PAP-Pen (hydrophobic slide marker). The sections were blocked with 3% goat serum (Jackson Immunosearch, USA) in PBS for 30 minutes.

The slides were dried without drying the sections. Primary rabbit antibody against human IGFBP-4 (anti IGFBP-4) was bought from Santa Cruz Biotechnology, USA. The primary antibody was tried in varying dilutions in 3% goat serum, namely 25, 50, 100, 200 and 400 dilutions. The sections were incubated with this antibody in different dilutions overnight at 4°C. 1/50 dilution yielded the optimum result at the end which was subsequently used.

The sections were washed three times, for 10 minute each, with freshly prepared PBS containing 0.1 % BSA. Secondary antibody fluorescent conjugate (Goat IgG (H+L) – F (ab)'2 antibody, PARIS, France) was diluted in 1/50 and 1/100 using 3% goat serum in PBS. 1/100 yielded a better result. After the addition of secondary antibody the slides were incubated at room temperature for 1 hour.

The slides were washed with PBS 3 times, for 5 minutes each. Nucleus was counterstained with either DAPI (1/1000 dilution) or Propidium Iodide containing mounting media (Vectone, UK). Then an anti-fading agent AF1 (Citiflour Ltd, Leicester, UK) was applied. A cover slip was mounted and fixed with nail polish. The slides were examined under a confocal microscope using fluorescent light and UV light for DAPI.

2.7.3 Western blot for IGF-IR

Primary rabbit polyclonal antibody (Santa Cruz, USA) was used in 1/200 dilution. The remaining steps were the same as for Bcl-2.

2.8 Statistics

Statistical analysis was carried out using Excel (Microsoft Office 2000, USA) and Graph Pad (Prism 4 for Windows 2003 version, Graph Pad software, USA) on a Windows based computer. All results are expressed as mean±standard error of mean. The unpaired student *t*-test was used for comparing two groups, and one way ANOVA was used for comparing three groups. Differences with P-value < 0.05 were considered significant.

Chapter 3 Effect of IGFBP-4 gene therapy on tumour characteristics and IGF system expression

3.1 Introduction

Recent evidence has shown that the insulin-like growth factor (IGF) system is involved in normal mammalian cellular growth, as well as in the development of several cancers [Durai et al., 2006;Giovannucci, 2001]. The IGF system comprises of two ligands (IGF-I and IGF-II), two main IGF receptors (IGF-IR and IGF-IIR), six IGF-binding proteins (IGFBP-4) and proteases. Many colon cancer cell lines, including HT-29, show a dose dependent proliferation in response to IGF-I and IGF-II [Guo et al., 1992;Zarrilli et al., 1994]. Studies have shown that any condition that increases the level of free IGF, such as acromegaly, will also increases the risk of colon cancer. IGF-I receptor is involved in tumour growth, without which tumour growth is reduced [Adachi et al., 2002]. At any one time most IGF-I and –II are bound to IGFBP, which modulate the bioavailability of the free IGF that induces cell growth. Among the IGFBPs, IGFBP-4 is secreted by almost all colon cancer cell lines, and it primarily functions as an inhibitory protein for colon cancer cells [Dai et al., 1997] by binding to both IGF-I and IGF-II with equal affinity [Zhou et al., 2003] [Singh et al., 1994a]. No previous studies available to date to demonstrate the effect of IGFBP-4 overexpression on colon cancers *in vivo*. The aim of this chapter was to explore the effect of IGFBP-4 gene therapy on subcutaneously induced colon cancers. The parameters assessed include tumour characteristics, i.e., tumour volume and weight, cell death, mitotic figures, expressions of IGFBP-4 and IGF-IR and animal body weight.

3.2 Material and methods

Mammalian expression vector pcDNA 3 (Invitrogen, Carlsbad, CA, USA) containing light chain myosin enhancer was used in this study, in which IGFBP-4 cDNA was inserted between *Eco*RI and *Kpn*I restriction sites, downstream of cytomegalovirus promoter. Plasmid DNA isolation, multiplication and purification were performed using the Endo Free Plasmid Maxi Kit (Qiagen, Crawley, UK). The presence of the IGFBP-4 insert and its reading frame were confirmed by cutting the plasmid DNA with *Eco*RI and *Kpn*I restriction enzymes and sequencing (MWG, Ebersberg, Germany).

Human colon adenocarcinoma HT-29 cells were cultured in McCoy's 5A medium enriched with glutamine (2 mM), 10% foetal calf serum and 1% penicillin and streptomycin at 37°C in an atmosphere of 5% CO_2. Only cells with >90% viability, as determined by Tryphan blue exclusion, were used. A subcutaneous cancer model was established by inoculating with 3 x 10^6 HT-29 cells in PBS per mouse under light enflurane general anaesthesia. The animals (n=6 each) also received plasmid (150 μg) containing IGFBP-4 cDNA (BP- 4 group), or plasmid of vector (control M), or just PBS (control P) at two different time periods, either on day 0 (early gene transfer model) or on day 8 (late gene transfer model). In the early gene transfer model, there were three groups (BP-4, control M and control P) and in the late gene transfer model, there were two groups (BP-4 and control M).

Four weeks after tumour induction, the animals were killed and tumour samples were obtained for histology and subsequent examination. Tumour volume (mm^3) was calculated using the formula (the shortest diameter) ^{2} × (the longest diameter) × 0.5

[Sawaoka et al., 1998]. Full details of plasmid purification and confirmation of its sequence, culture of HT-29 cells, induction of tumour in animal model and tumour harvesting were described previously in chapter 2 under materials and methods.

3.3 Results

3.3.1 Tumour characteristics

All the animals developed subcutaneous tumour on day 10 of the experiment, in both early and late gene transfer models, following inoculation with HT-29 cells. The tumours were well localised without any visible metastases. In the late gene transfer model (LGTM), the mean tumour volume was higher in the BP-4 group when compared with the control group, but it was not statistically significant (figure 3.1). Tumour weight was similar in both groups (not shown).

In the early gene transfer model (EGTM), both tumour weight and volume showed a reduction in animals which received plasmid treatment when compared to control P group tumours, although there was no statistical difference in the final tumour weight (1.70±0.17 vs 0.98±0.16 vs 1.19±0.25; control P vs control M vs BP-4; P= 0.06) (figure 3.2) or final tumour volume (2060.25±379.62 vs 829.99±194.81 vs 1007.71±246.67; Control P vs Control M vs BP-4; P = 0.69) (figure 3.3) between control M and BP-4 group.

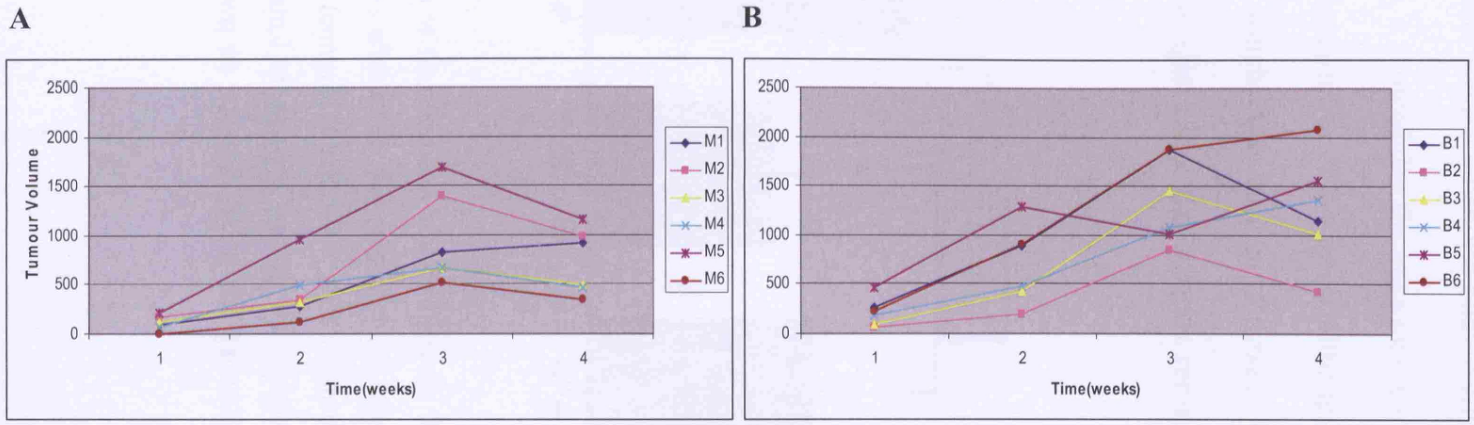


Figure 3.1 Scatterplot showing volumes of BP-4 and control group tumours (mm^3) in the late gene transfer model up to 3 weeks following a single injection of gene constructs with and without BP-4. Fig A represent tumours of control group and Fig B represent tumours of BP-4 group; week 1 = before plasmid treatment, week 2, 3 and 4 = 1, 2 and 3 weeks after plasmid treatment. The above graph shows that the tumours slightly shrink in size from the second week onwards.

3.3.2 Animal body weight

In the late gene transfer model, when the body weight of the animals were plotted over a period of time, it showed that the mice maintained their weight throughout the experimental period irrespective of the group, although the BP-4 group animals had a higher average body weight than the control group. Also, in the early gene transfer model, the average weight of all the animals remained the same throughout the experiment.

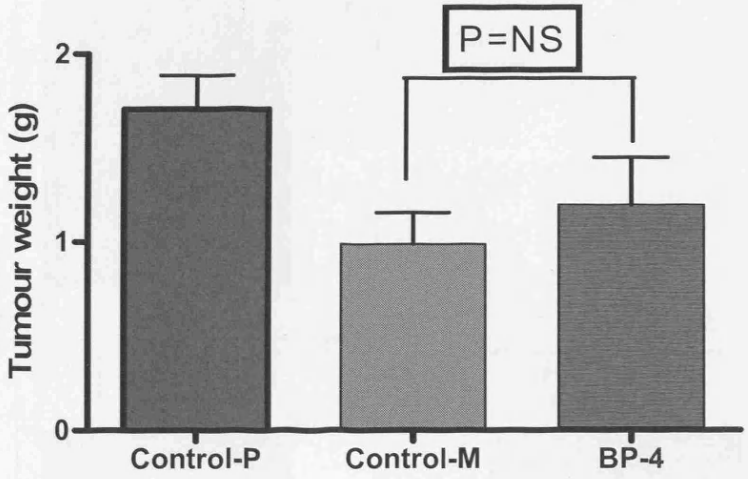


Figure 3.2 Comparison of final tumour weights between the three groups in the early gene transfer model, 4 weeks after single administration of saline (control P) or gene constructs with or without BP-4 (control M and BP-4 group respectively). Animals which received plasmid (BP-4 and control M) showed less tumour weight than those which received only PBS. Values are shown as mean±SEM of six animals in each group *(Key: NS=non significant).*

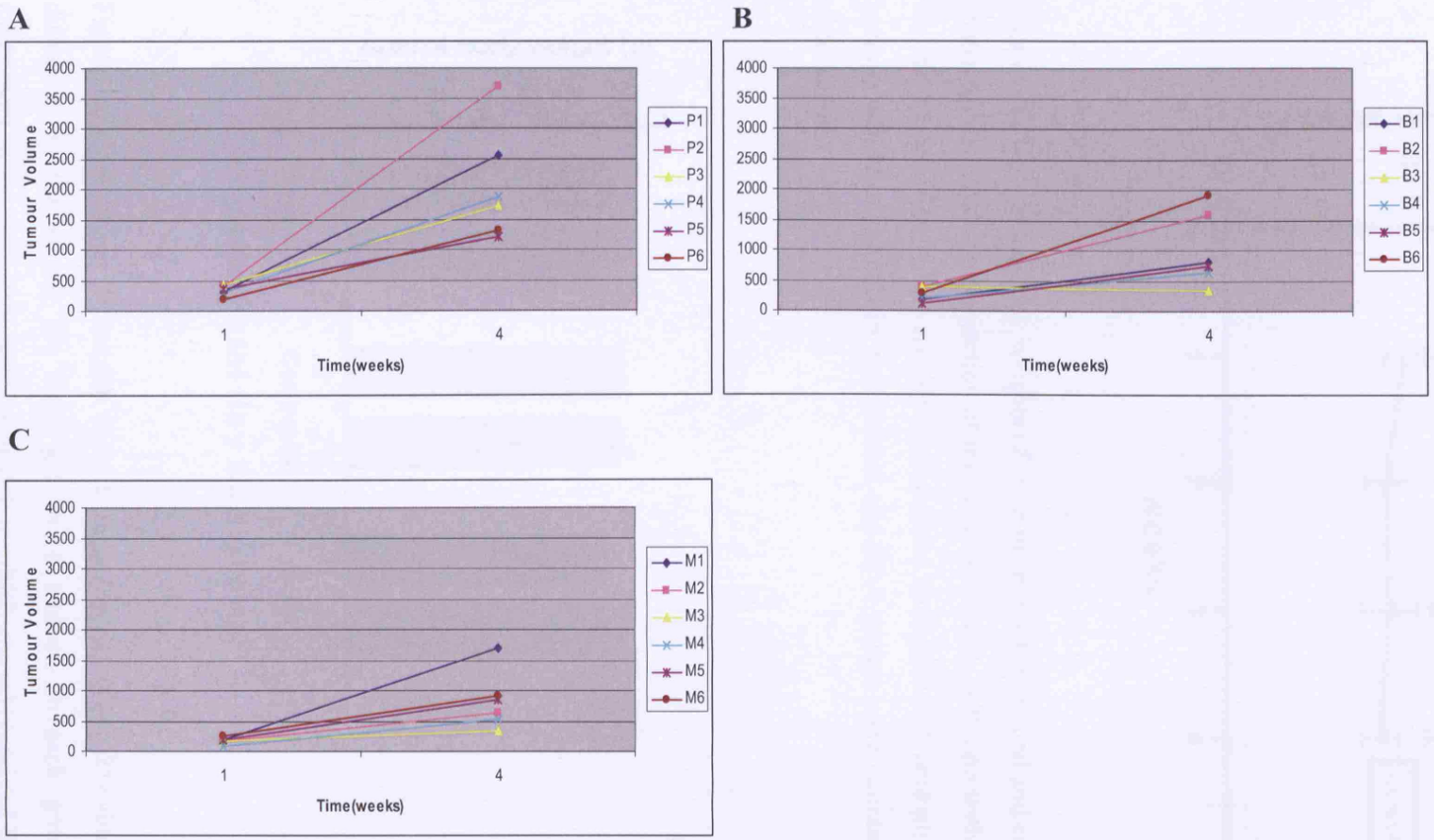


Figure 3.3 Scatterplot showing tumour volumes of three different groups in the early gene transfer group. Fig A represents control P tumours, Fig B represents BP-4 group and Fig C represents control M tumours. After plasmid therapy (Control M and BP-4) tumours showed a reduction in volume when compared to control P. (Key: control P - animals without any plasmid therapy, control M - animals which received control plasmid, BP-4 –animals which received IGFBP-4 gene therapy).

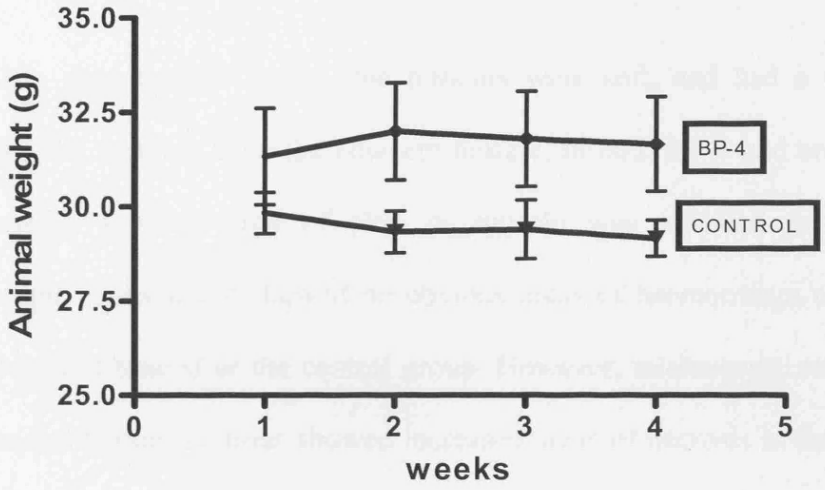


Figure 3.4 Comparison body weights of nude mice in BP-4 treated and control group up to 3 weeks following a single injection of the gene constructs, with and without BP-4 (late gene transfer model). The values are depicted as mean mean±SD of 6 animals in each group. The average weight of animals in both control and BP-4 groups were maintained throughout the experiment.

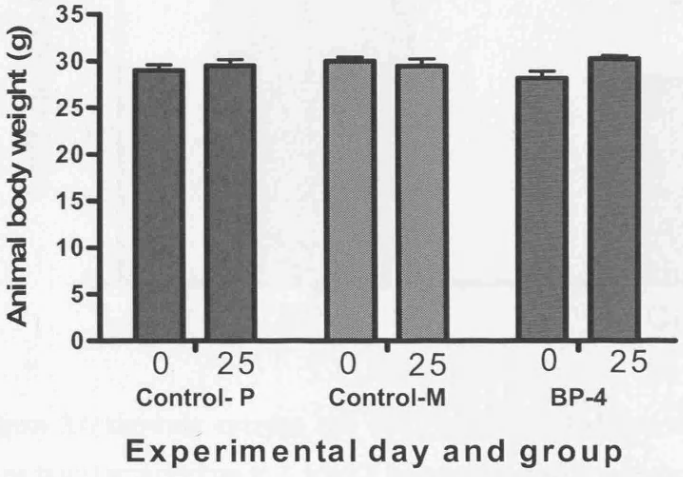


Figure 3.5 Comparison of weights of nude mice (on day 0 and 25) showing no significant difference in the among the three groups (6 animals in each group) throughout the experiment in the early gene transfer model, in which animals received BP-4 gene or control plasmid or PBS on day '0' (*key: P – animals without plasmid therapy, M - animals which received control plasmid, BP-4 – animals which received IGFBP-4 gene)*

3.3.3 Tumour histology

In the late gene transfer model, the tumours were soft, and had a false capsule separating the tumours from the adjacent muscle, in both BP-4 and control groups. Occasionally, a small area of skin or muscle was attached to the tumour. Macroscopic examination showed no obvious areas of haemorrhage or necrosis in either the BP-4 treated or the control group. However, microscopic examination of H&E stained tumour sections showed increased areas of necrosis in the BP-4 group when compared with the control group. The BP-4 treated group showed a considerably higher cell death score when compared with control (2.33 ± 0.33 vs 1.33 ± 0.49) (figure 3.6) but the difference was not statistically significant (P=0.12).

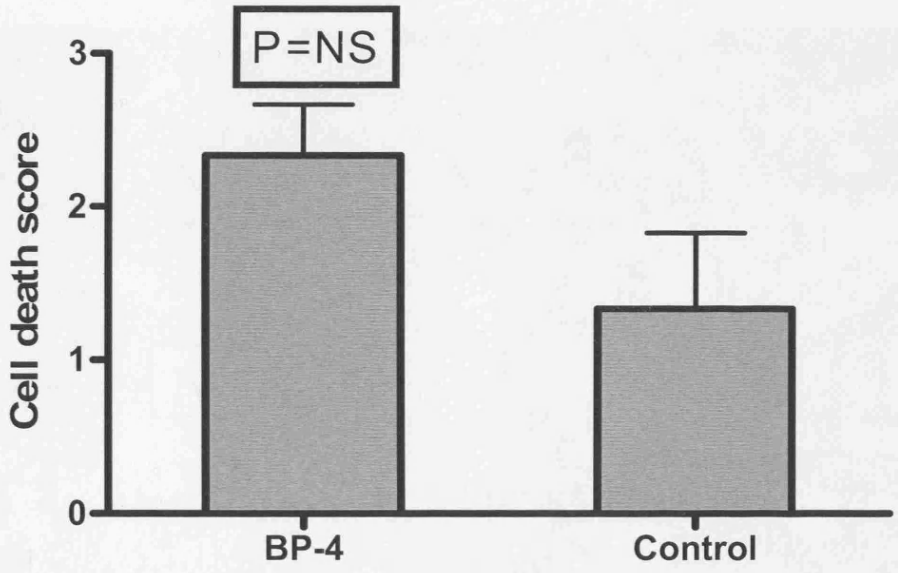


Figure 3.6 showing average cell death score in BP-4 and control group tumours of the late gene transfer model up to 3 weeks following a single injection of the gene construct with and without BP-4. In this experiment a subcutaneous cancer was induced first in nude mice with HT-29 cancer cells and gene construct / control was administered as peritumoral injection one week post tumour induction. Values are expressed as mean ± SEM of 6 animals in each group. (*Key NS= not significant)*

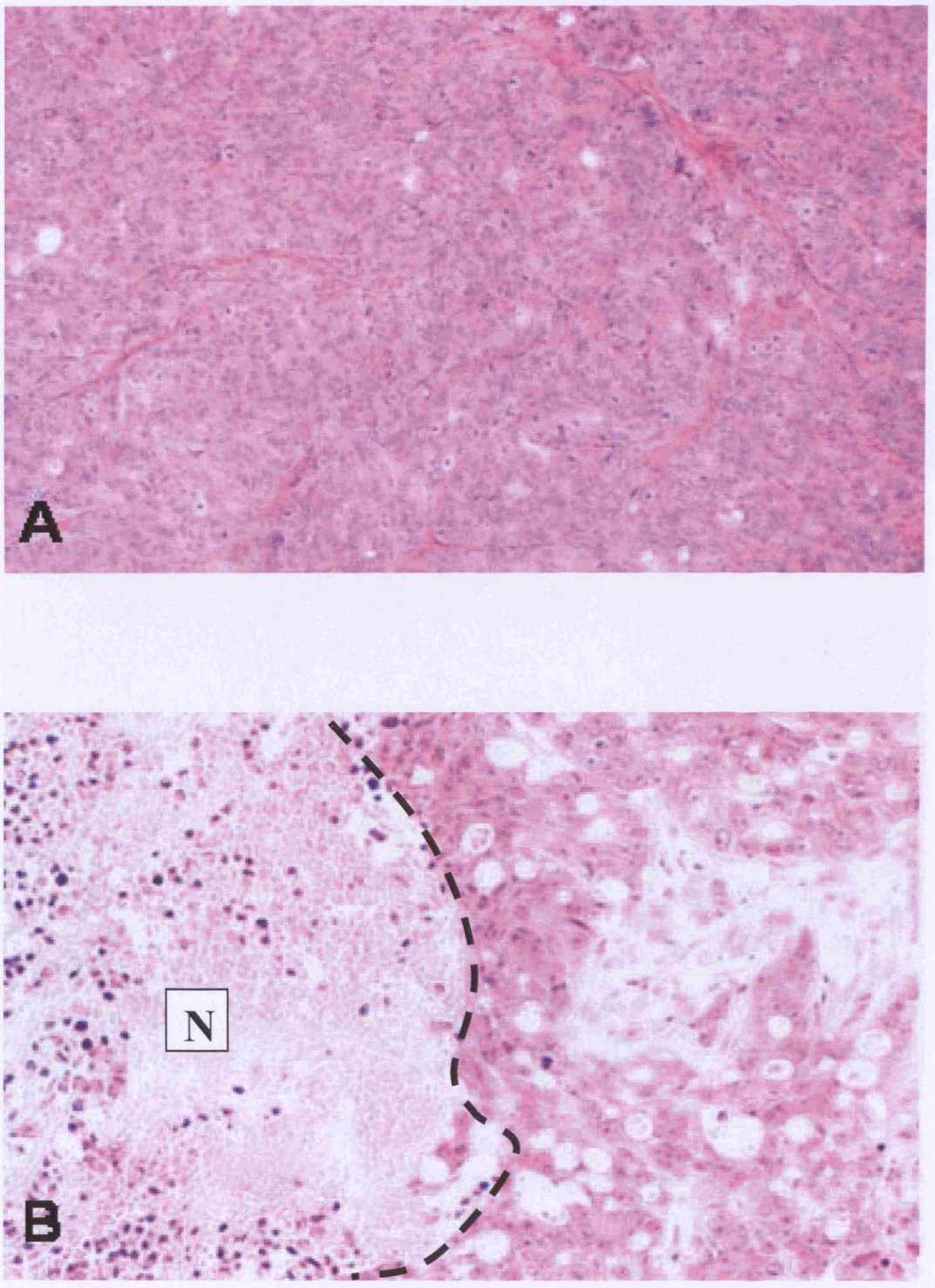


Figure 3.7 Photomicrographs (x100) showing morphological changes after administration of control plasmid (A) and plasmid vector with BP-4 insert (B) in subcutaneous tumours of mouse induced with HT-29 in late gene transfer group. Large necrotic areas and loss of intact cells are seen after the administration of IGFBP-4 gene when compared with control. (N= areas of cell death)

In the early gene transfer model, the tumour morphology was similar to the late gene transfer model. There was an attempted glandular formation with areas of cell death. There was no local or distant spread of cancer. The mean area of cell death was significantly higher in BP-4 treated tumours, when compared with the other two control groups (2.20±0.22 vs 1.76±0.23 vs 0.66±0.14, BP-4 vs Control M vs Control P, P=0.0007). Figures 3.8, 3.9 and 3.10 show the representative histology of tumours after H&E staining in various groups, and figure 3.11 shows the cell death score.

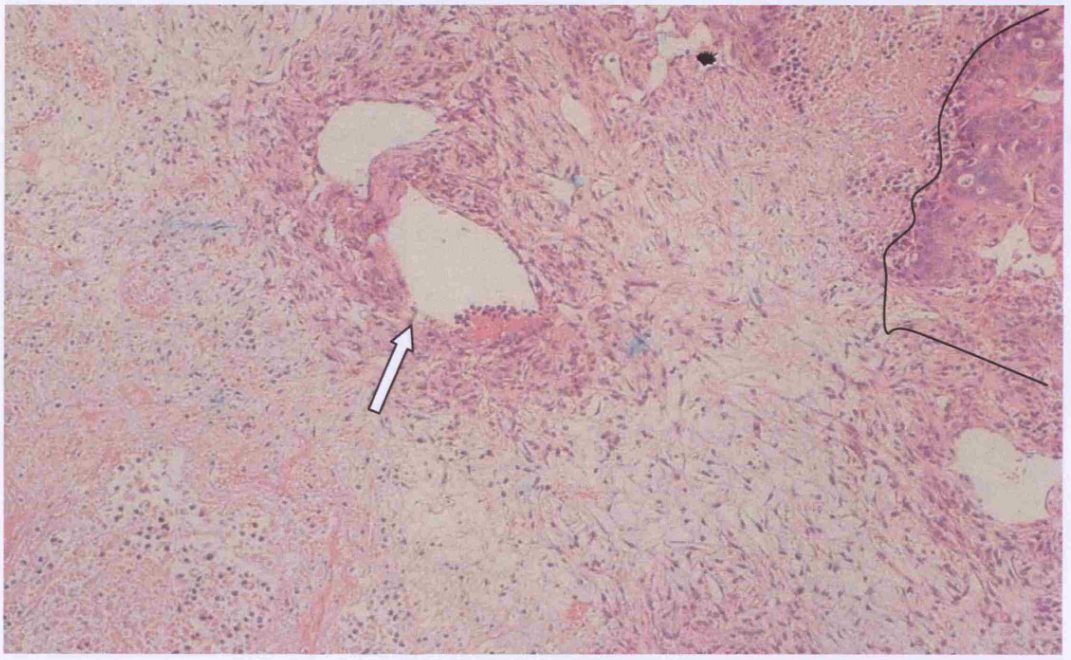


Figure 3.8 Photomicrograph showing H&E staining of subcutaneous tumour from BP-4 group of the early gene transfer model (×100). In this experiment a subcutaneous cancer was induced in nude mice with HT-29 cancer cells and gene construct / control was administered on day '0' at the same time as tumour induction. Areas of cavitation (arrow) and necrosis are present when compared to control groups which are shown on the next page.

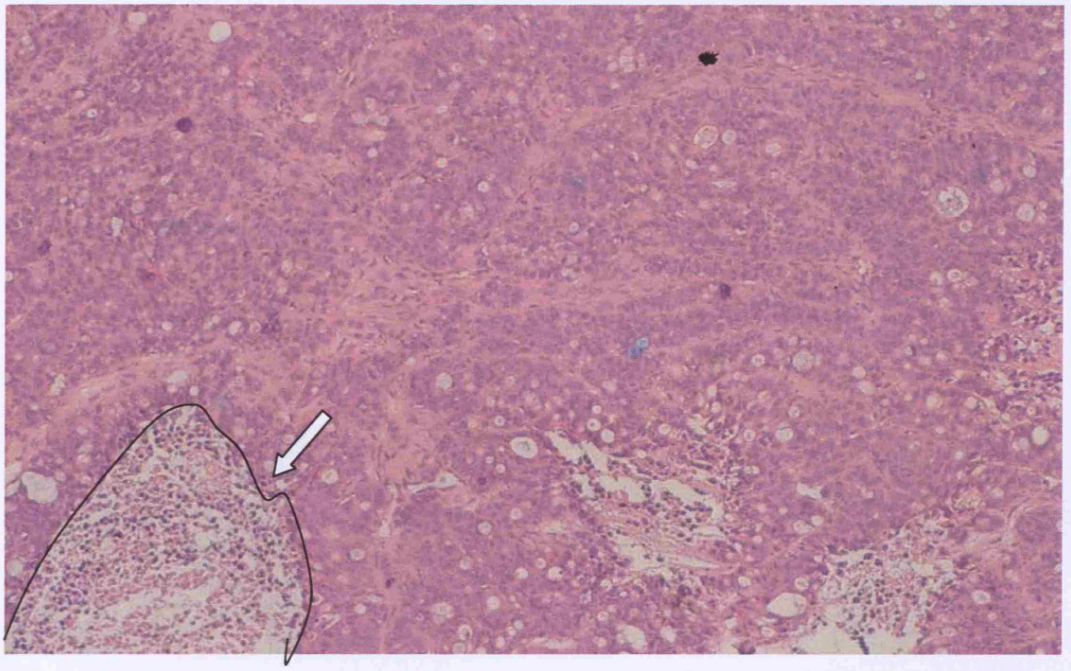


Figure 3.9 showing H&E staining of subcutaneous tumours of nude mice from control M group of the early gene transfer model (×100) which were treated with control plasmid on day 0. There are some areas of cell death (arrow).

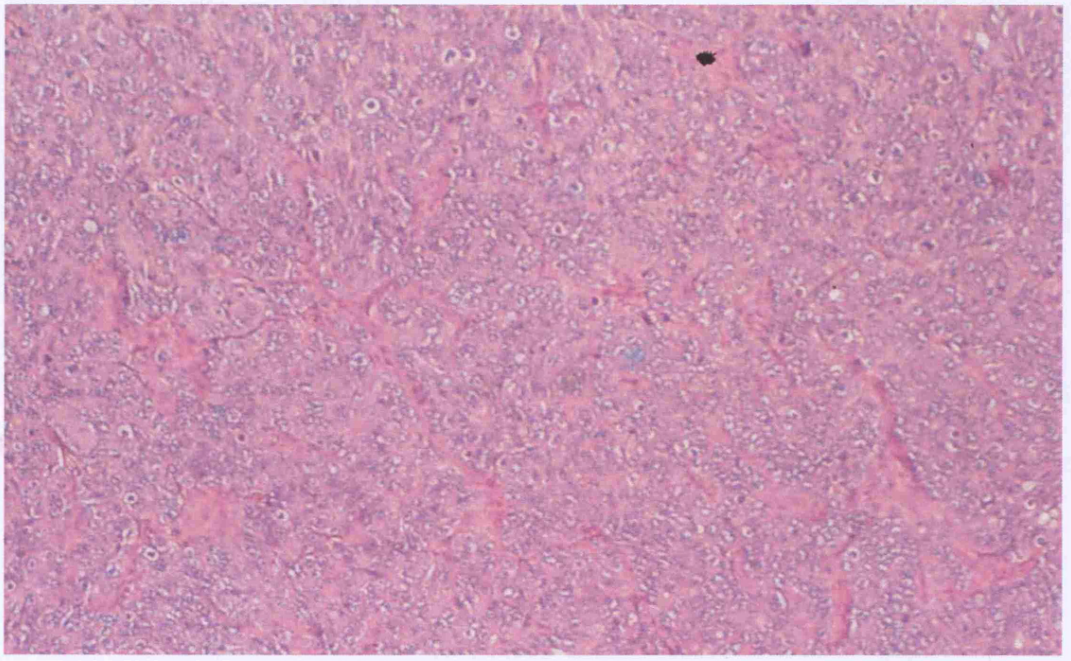


Figure 3.10 showing H&E staining of subcutaneous tumours of nude mice from control P group of the early gene transfer model (×100) which did not receive any plasmid therapy. There is no obvious area of cell death.

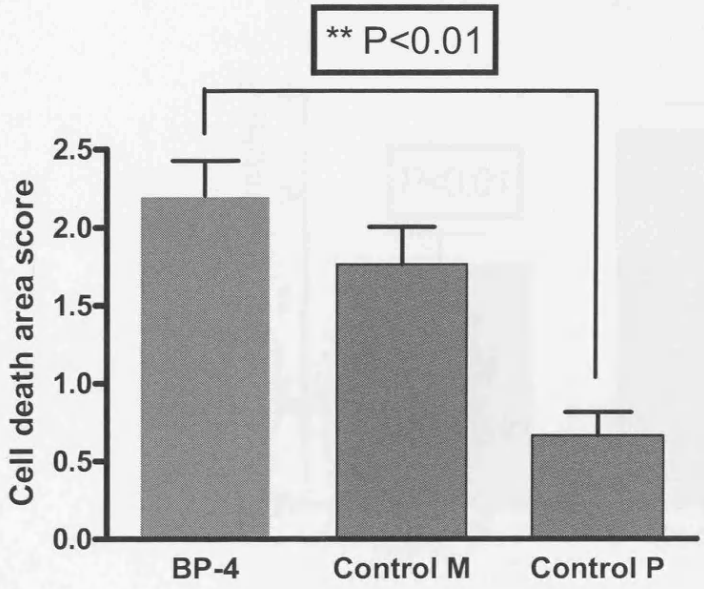


Figure 3.11 Tumours of the early gene transfer model showing significantly higher cell death score in the BP-4 group compared to the control groups (P<0.01). All the animals received a single administration of saline or gene construct with and without BP-4 along with colorectal cancer cells. Cell death score was calculated after H&E staining of paraffin sections of tumour samples. Results are shown as mean±SEM of 6 animals in each group. (*Key: P – animals without plasmid therapy, M - animals which received control plasmid, BP-4 – animals which received IGFBP-4 gene.)*

3.3.4 Mitosis

In the late gene transfer model, the average numbers of mitotic figures were significantly higher in tumours of control group when compared with the BP-4 group (3.61± 0.27 vs 2.31± 0.32 Control vs BP-4, P=0.0067) (figure 3.12).

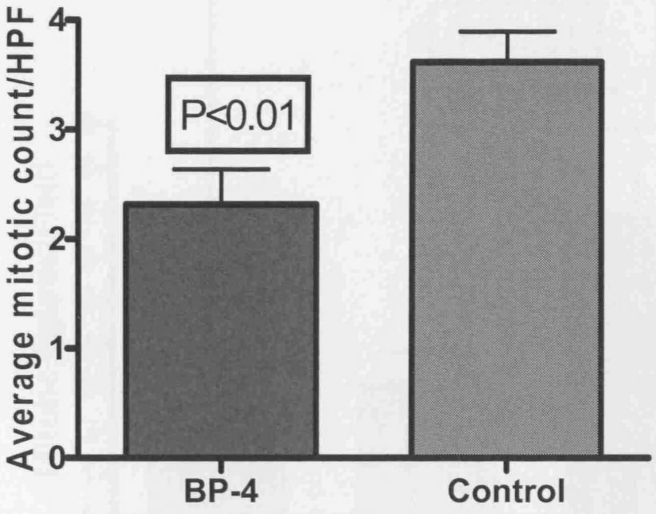


Figure 3.12 Comparison of the average number of mitotic figures of subcutaneous tumours of the late gene transfer model per high power field (HPF) (400 magnification) between control and BP-4 groups (based on counting from 10 HPF). In this late gene transfer model, animals received gene constructs with or without IGFBP-4 cDNA on day 8 as peritumoral injection when tumours were palpable. Values are expressed as mean ± SEM of 6 animals each group, $P< 0.01$ control vs BP-4.

In the early gene transfer model, the effect on mitoses was similar to the late gene transfer model. The average numbers of mitotic figures per field were lower in the BP-4 group tumours than the control group (2.03±0.53 vs 3.13±0.99 vs 7.00±1.72, BP-4 vs Control M vs Control P, P=0.03) (figure 3.13).

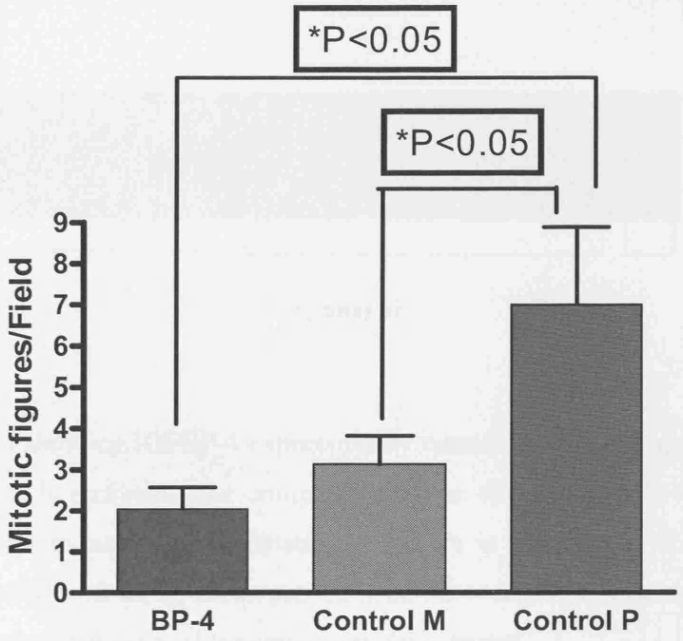


Figure 3.13 showing average numbers of mitotic figures per tumour section per field in the tumours of the early gene transfer model in which the mice received gene construct with or without BP-4 or none at the same as tumour induction with HT-29 cells. Mitosis was calculated after H&E staining of paraffin sections of tumour sample. The average numbers of mitotic figures were decreased after administration of BP-4 gene when compared with control groups ($P<0.05$). Values are expressed as mean ± SEM of 6 animals in each group.

3.3.5 IGFBP-4 expression

Expression of IGFBP-4 by tumour tissues was detected by Western blot analysis (figure 3.14). In the late gene transfer model, densitometry analysis showed a statistically significant increase in the IGFBP-4 protein levels in the cancer tissue after BP-4 gene therapy when compared with the control group (15.70 ± 1.31 vs 11.61 ± 1.27, $P<0.05$ vs Control) (figure 3.15). On immunolocalization, the IGFBP-4 was found both within the cancer cells and in the interstitial area. Within the cell, it was mainly localised in the cytoplasm. Animals that received the IGFBP-4 gene showed a higher expression of IGFBP-4 in the tumour, both in the cytoplasm and interstitium, when compared with the tumours of the control group. Two figures representing IGFBP-4 immunostaining are shown in the appendix.

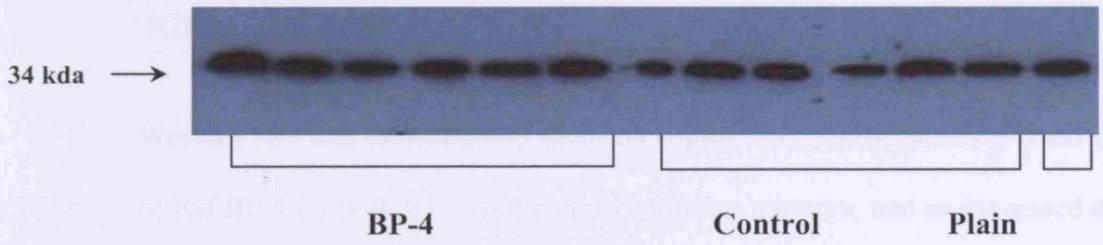


Figure 3.14 Western blot showing IGFBP-4 expression by tumours of the late gene transfer model in which the animals received gene constructs with or without IGFBP-4 cDNA as peritumoral on day 8 after induction of subcutaneous cancers in nude mice. *(Key: BP-4 - tumours receiving plasmid with BP-4 construct, control M – tumours receiving control plasmid therapy and Plain - tumours without any plasmid treatment)*

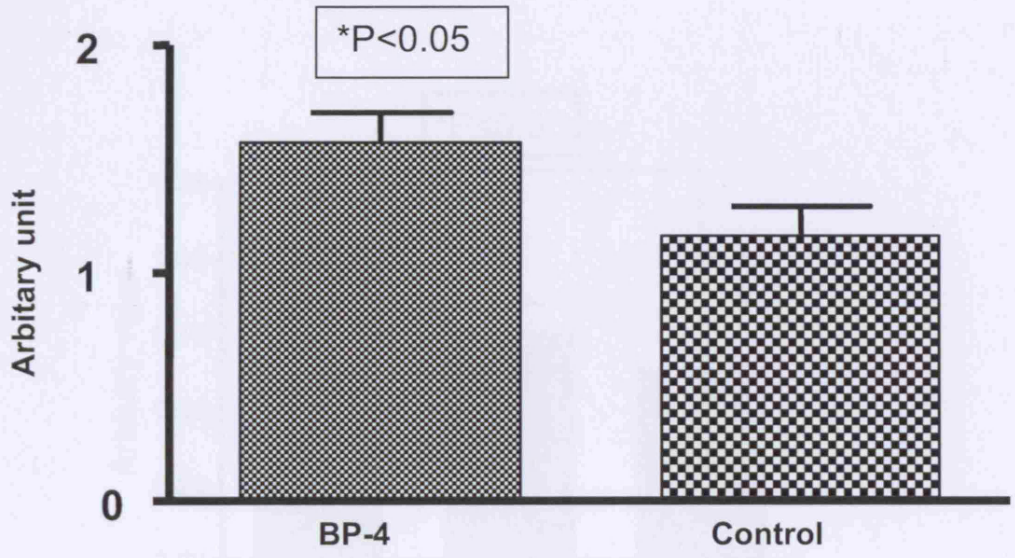


Figure 3.15 Quantification of IGFBP-4 expression by densitometry analysis which showed a significant increase in expression of IGFBP-4 after IGFBP-4 gene therapy ($P<0.05$ BP-4 vs control). (Key: BP-4 = IGFBP-4; C = Control; Plain = no treatment)

Early gene transfer model

IGFBP-4 expression

Western blot and densitometry analysis (figure 3.16) showed a decreased expression of IGFBP-4 by both BP-4 and control M group tumours, and an increased expression by Control P group (0.63±0.03 vs 0.75±0.09 vs1.04±0.06, BP-4 vs control M vs Control P, P=0.002).

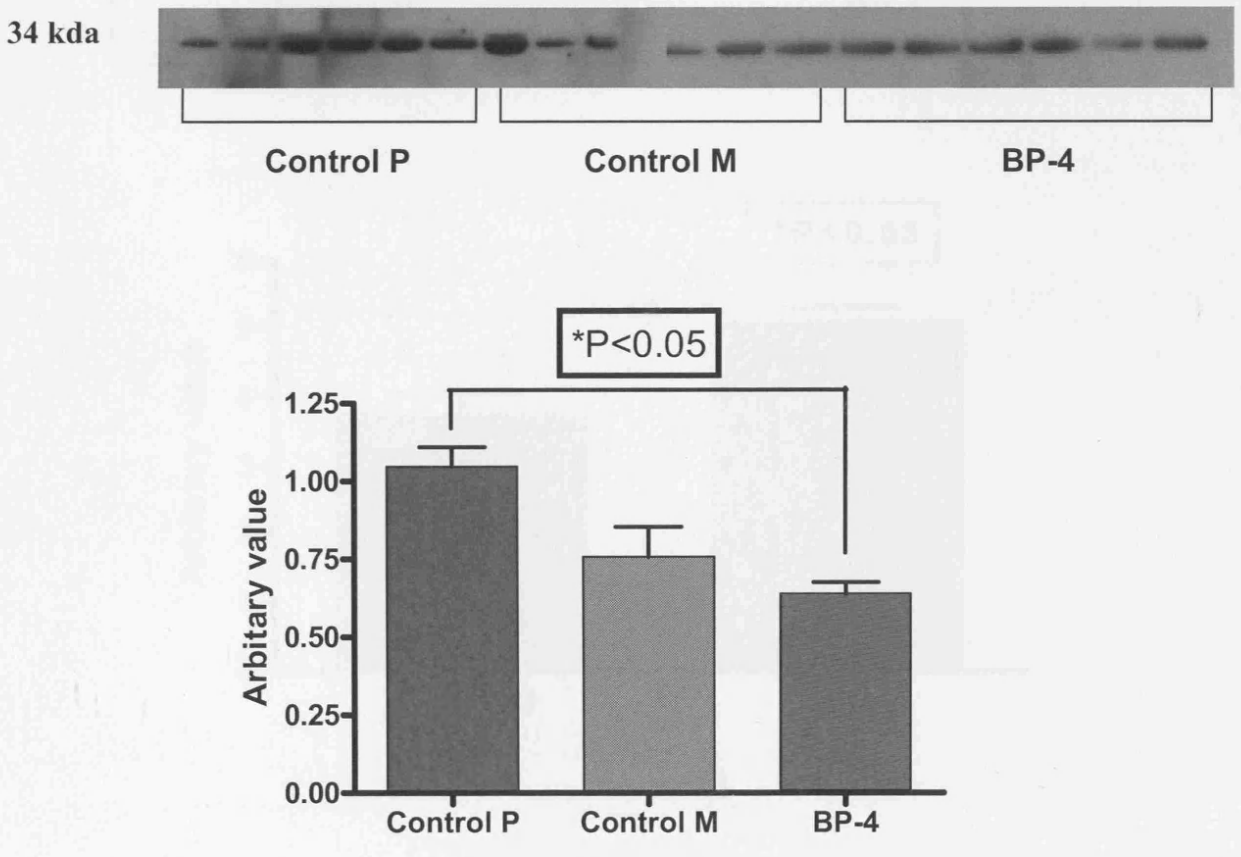


Figure 3.16 Western blot for IGFBP-4 expression (a) and densitometry analysis (b) showing higher expression of IGFBP-4 by Control M than the other two groups (P<0.05). In this experiment subcutaneous cancers were induced in nude mice with HT-29 and gene constructs with or without BP-4 was administered at the same time *(Key: control P - tumours without any plasmid treatment, control M – tumours receiving control plasmid therapy, BP-4 - tumours receiving plasmid with BP-4 construct)*

3.3.6 IGF-IR expression (Late gene transfer model)

Western blot showed increased IGF-IR levels (5.08 ± 0.21 vs 3.73 ± 0.31; BP-4 vs Control; P<0.01) in the BP-4 group tumours compared with the control group (figure 3.17).

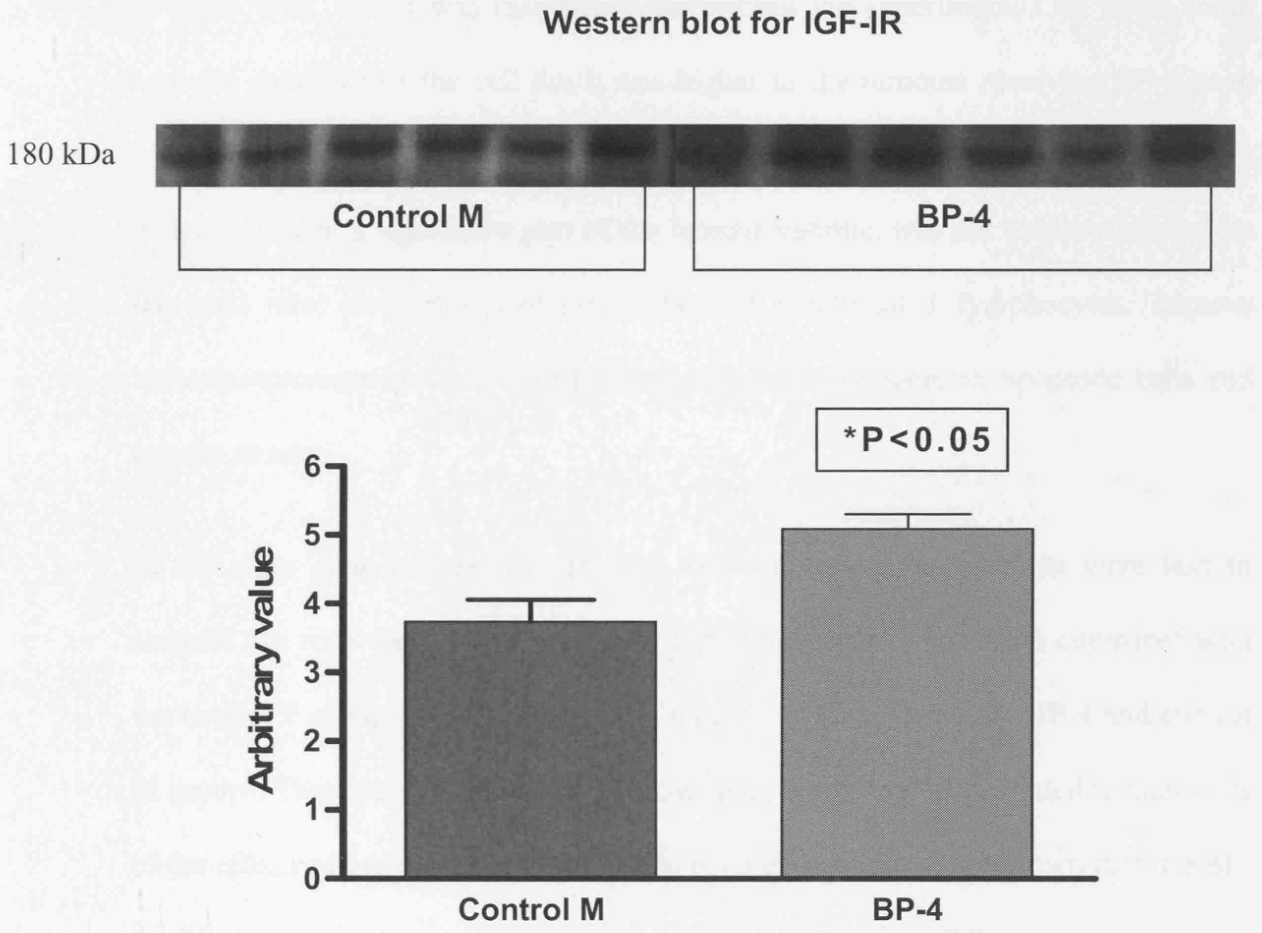


Figure 3.17 Western immunoblot for IGF-IR and densitometry analysis of tumours of the late gene transfer model in which animals received a single injection of gene construct with and without BP-4. Values are shown as mean± SEM of 6 animals per group. BP-4 group tumours showed a higher average IGF-I receptor expression when compared with the control group tumours (P<0.05).

3.4 Discussion

Tumour volume and histology

In the late gene transfer model, the mean tumour volume of the BP-4 group was higher even before the plasmid administration, and it was maintained throughout the experiment. The mean weight of animals treated with BP-4 plasmid was also higher to begin with, and it was maintained throughout the experiment. Cell death score analysis showed that the cell death was higher in the tumours receiving BP-4 gene construct. This may mean that the number of dead cells (apoptotic and necrotic) which represent a significant part of the tumour volume, was not totally removed by the nude mice circulation because of lack of functional T lymphocytes. Tumour volume represents not only proliferating cells but also necrotic, apoptotic cells and tumour stroma.

In the early gene transfer model, both tumour volume and weight were less in animals that received plasmid treatment (BP-4 and control M) when compared with the control P group, although there was no difference between the BP-4 and control M groups. These results show that macroscopic parameters are not reliable indicators of the effects of gene transfer. Previous *in vitro* experiments have shown that IGFBP-4 inhibits proliferating and growth-promoting actions of the IGFs in both malignant and normal cells [Zhou et al., 2004;Singh et al., 1996;Singh et al., 1994a]. Results of our experiments support these findings. Histological examination of the IGFBP-4 treated tumours, showed a higher cell death score and a lower mitotic count per field, in both early and late gene transfer models.

Components of the IGF system

IGFBP-4 acts mainly by sequestering the IGFs and is a very important inhibitory binding protein of the IGF system. IGFBP-4 mRNA expression and secretion by various tissues are influenced by IGFBP protease [Byun et al., 2000;Durham et al., 1995], nutrition [Hallberg et al., 2000], trauma [Demori et al., 2000], several growth factors and hormones [Sheikh et al., 1993]. The IGFBP-4 mRNA expression is not necessarily a good indicator of the amount of IGFBP-4 protein associated with the cells. Although several types of cancer cells express IGFBP-4 [Bostedt et al., 2001;Ceda et al., 1991;Yi et al., 2001], and the role of IGFBP-4 has been widely studied in breast cancer, the results are controversial. *In vitro* and *in vivo* studies showed that overexpression of IGFBP-4 is inhibitory to many cancer cells [Singh et al., 1994a] [Damon et al., 1998]. However, there are exceptions, and reduced expression of IGFBP-4 may not increase cell growth in some cancers, for example, prostatic cancer [Drivdahl et al., 2001]. It has been shown that anti-sense inhibition of IGFBP-4 may confer growth advantage [Dai et al., 1997], while overexpression of IGFBP-4 may not be inhibitory to some colon cancer cells. To date, there are only two studies available in prostate cancer [Damon et al., 1998;Drivdahl et al., 2001] that have shown the effect of IGFBP-4 *in vivo*. Apart from the one study done by Damon et al, currently there is no *in vivo* evidence available to demonstrate that IGFBP-4 overexpression is inhibitory to cancers. In our experiment, the IGFBP-4 protein expression of the tumour was clearly increased in the late gene transfer model. There was an associated increase in IGF-IR levels in BP-4 treated group tumours. However, IGFBP-4 was not increased in BP-4 treated tumours of the early gene transfer model, although skeletal muscles of BP-4 group showed an increased IGFBP-4 expression. These results indicate that pre-existing tumour may be a

prerequisite for IGFBP-4 gene transfer, and there exists a feed back system, whereby the colon cancer cells increase the IGF-IR levels to overcome the decreasing levels of free IGFs.

In summary, we found that the IGFBP-4 gene therapy when delivered locally decreased mitosis and increased necrosis and apoptosis of subcutaneous cancer, although within the time period studied, the therapy did not significantly decrease the tumour volume. It was therefore important to know whether there had been any associated increase in apoptosis, and if so what was the mechanism. The next chapter will address these questions.

Chapter 4 Effect of IGFBP-4 gene therapy on apoptosis of the cancer cells

4.1 Introduction

Apoptosis is influenced by various intracellular proteins and enzymes. Two important proteins which influence apoptosis are B-cell CLL/lymphoma 2 (Bcl-2), which is an anti-apoptotic protein, and Bcl-2-associated X protein (Bax) which promotes apoptosis. Bax exists in an inactive state in the cytoplasm of many cells. Secondary to various stimuli, Bax protein undergoes conformational changes, and translocates to mitochondrial membranes, where Bax releases cytochrome c [Guo et al., 2003]. Cytochrome c is normally found loosely associated with the inner membrane of the mitochondria. It catalyzes several reactions such as hydroxylation and aromatic oxidation and is an intermediary for apoptosis. Experiments show that an increase in the ratio of Bax/Bcl-2 proteins often proceeds apoptosis [Bianchi et al., 2003;Giannakakou et al., 2001], and mutations of these genes are often associated with cancer [Meijerink et al., 1995;Ouyang et al., 1998;Ohtani et al., 2000]. IGFs are often overexpressed in cancers and are anti-apoptotic [Kang et al., 2003] in action. The IGFs influence the levels of Bax and Bcl-2 [Yamamura et al., 2001] and thereby influence apoptosis [Wang et al., 1998b]. IGF-I not only downregulate the Bax expression [Hong et al., 2001] but also prevent its translocation to mitochondria [Ness et al., 2004], inhibits the activation of caspase 3 [Linseman et al., 2002], and release of cytochrome c from the mitochondria [Linseman et al., 2002]. So when the IGFBP-4 is overexpressed the level of free IGF bioavailability decreases and one can expect them to increase apoptosis at least in the local tissues. The aim of this

experimental work was to assess the effect of IGFBP-4 gene transfer on the apoptosis, Bax and Bcl-2 proteins expression.

4.2 Materials and methods

The animal model and tumour harvesting are described in chapter 2 under the section materials and methods as well as in the earlier chapter. Apoptosis was determined by terminal deoxynucleotidyl transferase–mediated dUDP nick end labelling (TUNEL) assay using Apotag-red kit (Serologicals corporation, Temecula, CA, USA) [Gavrieli et al., 1992] and transmission electron microscopy. Frozen sections (10 μm) of tumour samples were fixed with 5% formalin for 10 minutes at room temperature. Then they were washed with PBS followed by immersing them in pre-cooled ethanol : acetic acid (2:1) for 5 minutes at -20°C. The protocol recommended by the manufacturer was followed. TdT enzyme and anti-digoxigenin rhodamine were used and the slides were protected from light. Finally, the slides were washed with PBS and mounted using glycerol based media AF-1 (Citiflour, Leicester, UK). Confocal microscope (C1, Nikon, Kanagawa, Japan) was used for evaluating apoptosis. Apoptotic cells were identified by red fluorescence, and the counting was carried out blindly. For quantification of apoptosis, 10 random fields were chosen for each sample at × 200 magnification [Bruns et al., 2000], and the average cell count was used. The counting was carried out blindly by two observers (R Durai and W Yang). The Apoptotic Index was calculated using the formula as described previously [Sawaoka et al., 1998] (i.e. Apoptotic index (%) = Apoptotic cell number/Total cell number ×100). The method of processing the tumour sections for electron microscopy was described previously. It was performed for the early gene transfer model only. The ultra thin sections (60-90 nm) were viewed and photographed using a Philips CM120 transmission electron microscope.

Bax and Bcl-2 levels were determined by Western blot. Rabbit anti-mouse Bcl-2 polyclonal antibody (Santa-Cruz Biotechnology, Santa Cruz, CA, USA) or rabbit anti-mouse Bax polyclonal antibody (Santa-Cruz Biotechnology, USA) both at 1/200 dilution were used, followed by incubation with secondary antibody conjugate with horse radish peroxidase (HRP) (Dako, Ely, UK) at 1/2000 dilution. The PVDF membrane that was used for electroblotting was illuminated with Super Signal West Dura Extended Duration Substrate (Pierce Biotechnology, Rockford, IL, USA), and exposed to X-ray film for 10 seconds (Fuji, Kanagawa, Japan).

4.3 Results

4.3.1 Apoptosis quantification by TUNEL assay

In the late gene transfer model, apoptotic cells were detected in considerable numbers in a diffusely scattered manner throughout the tumour tissue, in those animals which received the IGFBP-4 gene. The tumours of animals in the control group (figure 4.1 b) showed fewer apoptotic cells when compared to tumours of those animals treated with IGFBP-4 plasmid (figure 4.1a). Apoptotic Index was significantly higher in tumours of the BP-4 group than control (36.67 ± 7.36 vs 7.07 ± 1.91, BP-4 vs control; P = 0.003) (figure 4.3).

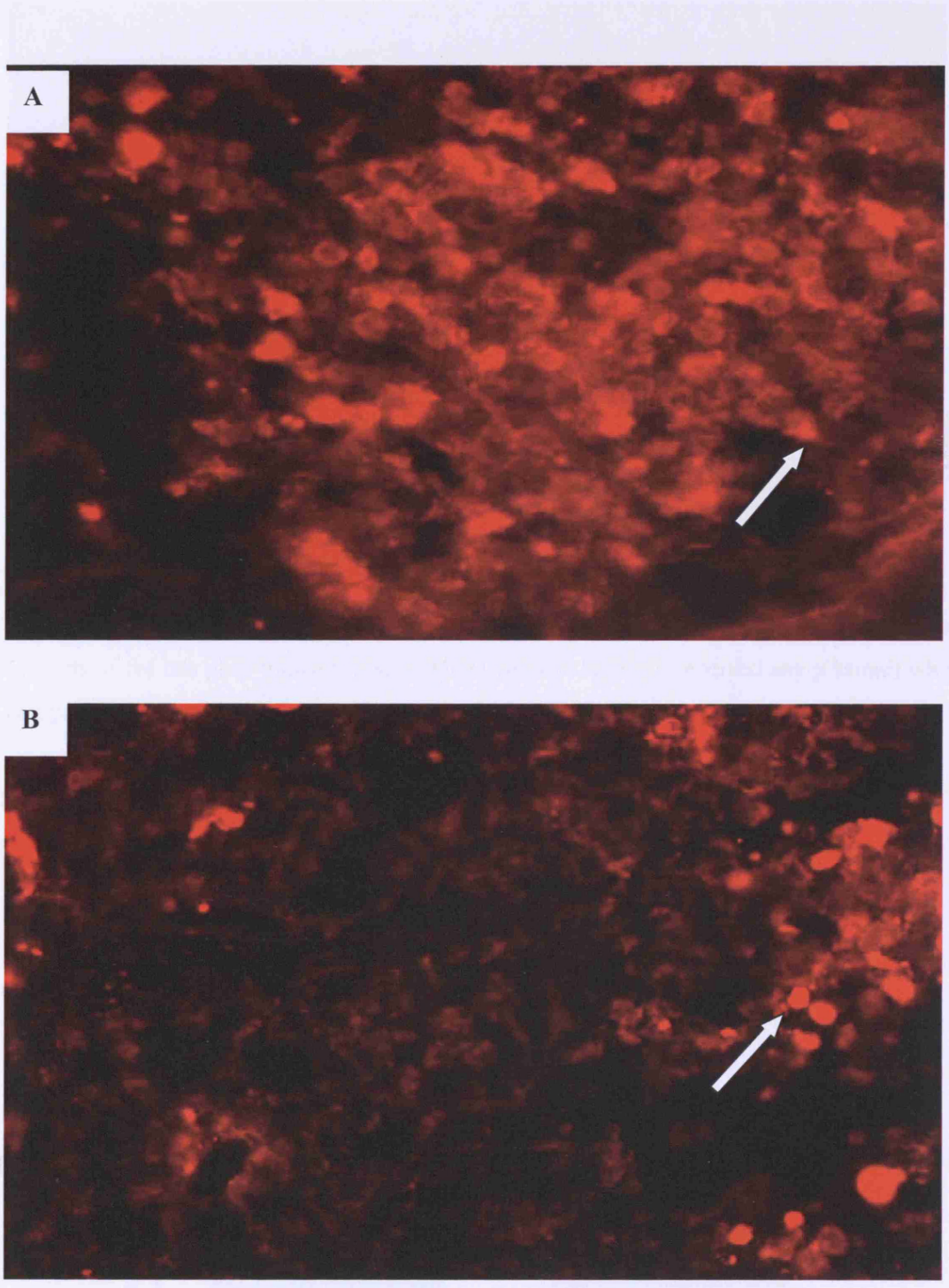


Figure 4.1 Representative photomicrographs showing apoptotic cells in BP-4 (a) and control (b) group tumours of the late gene transfer model, in which animals received a single dose of gene construct with or without IGFBP-4 as peritumoural injection on day 8 after the development of subcutaneous tumours. Apoptotic cells were detected by red fluorescence and nuclear morphology (arrow) (× 200).

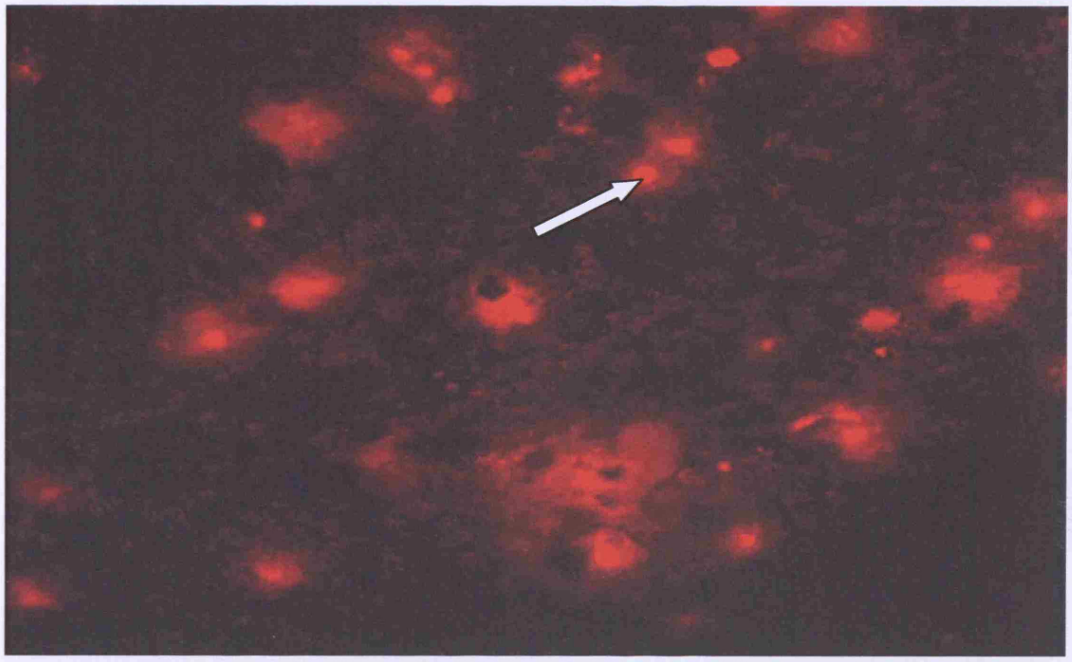


Figure 4.2 Photomicrograph showing fewer numbers of apoptotic cells of control P group tumours of the late gene transfer model (where animals have not received any plasmid) when compared to the other two group tumours (× 200).

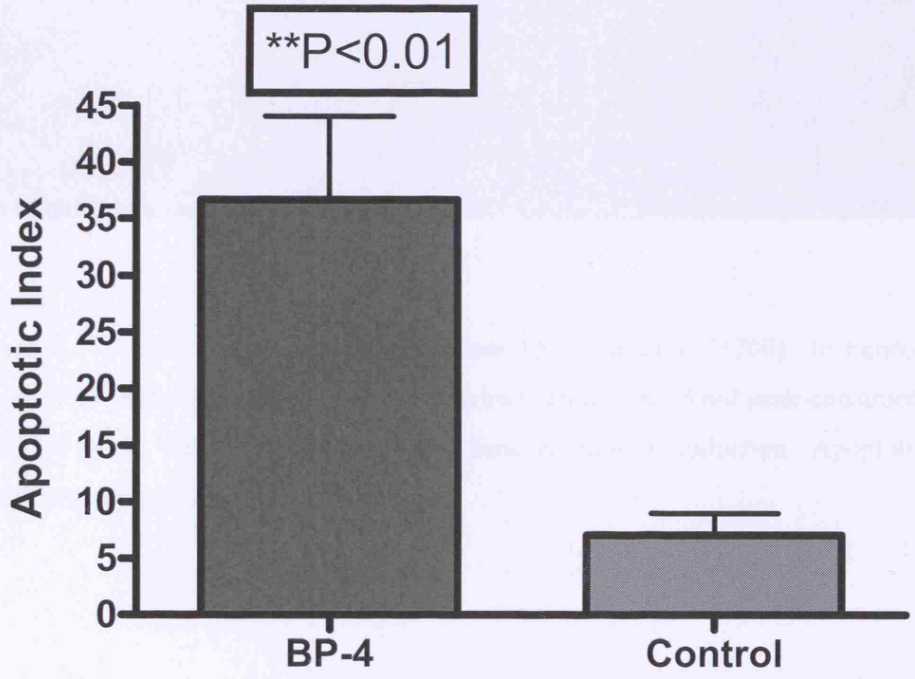


Figure 4.3 Comparison of apoptotic index (average apoptotic cells per 100 tumour cells per field) between BP-4 and control group tumours of the late gene transfer model, in which the animals received gene construct with or without IGFBP-4 gene as peritumoural injection on day 8. Values are expressed as mean ± SEM, 6 animals in each group, P< 0.01 BP-4 vs control.

In the preventive (early gene transfer) model, TUNEL staining showed higher numbers of apoptotic cells in tumours of BP-4 group than control group (figures 4.4 and 4.5). Apoptotic Index is plotted in figure 4.6. It shows that the BP-4 group tumours have higher numbers of apoptotic cells for every 100 cells (11.47± 1.51 vs 3.59±0.17 vs 4 .47±0.55, BP-4 vs Control M vs Control P, P=0.0002).

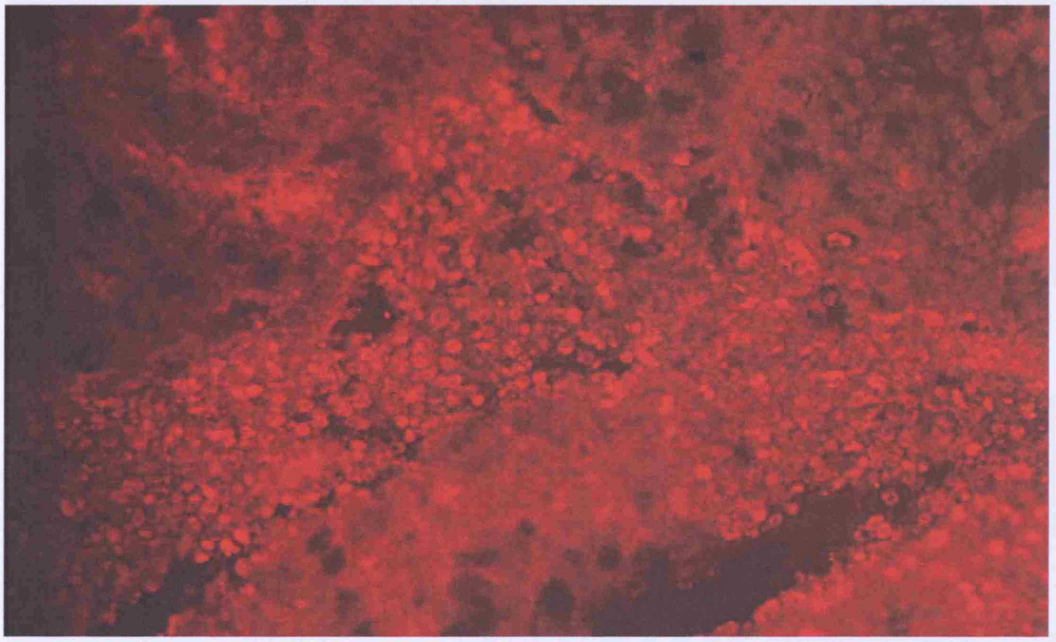
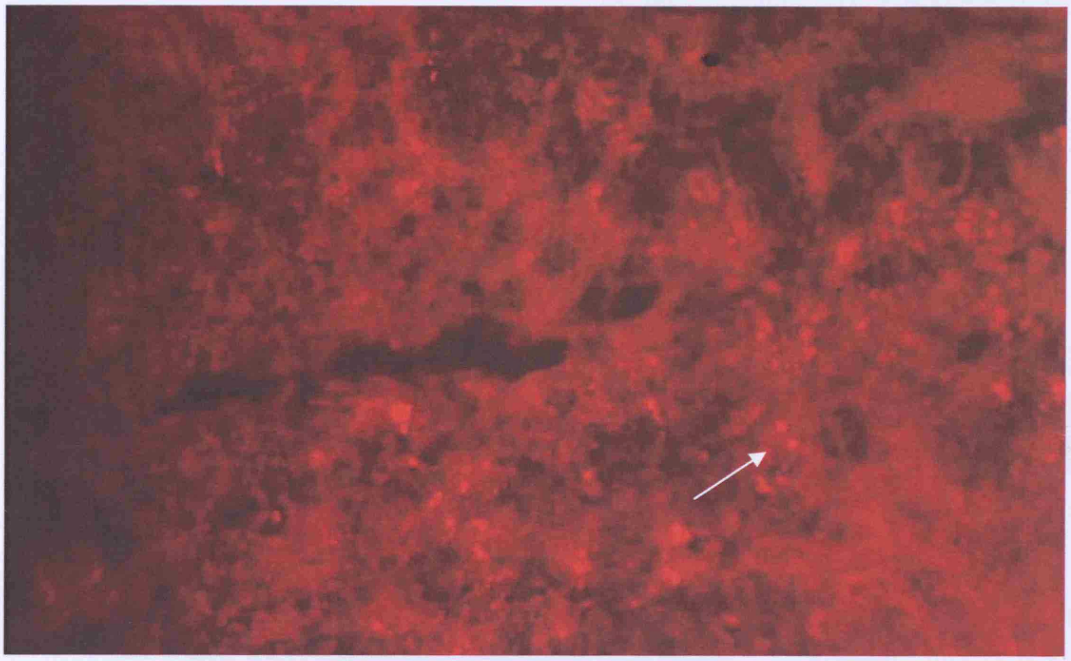


Figure 4.4 TUNEL assay showing numerous apoptotic cells (×200) in tumours of BP-4 group of the early gene transfer model in which animals received gene construct containing IGFBP-4 gene, on day 0, at the same time as tumour induction. Apoptotic cells are identifiable by red immunofluorescence.

A



B

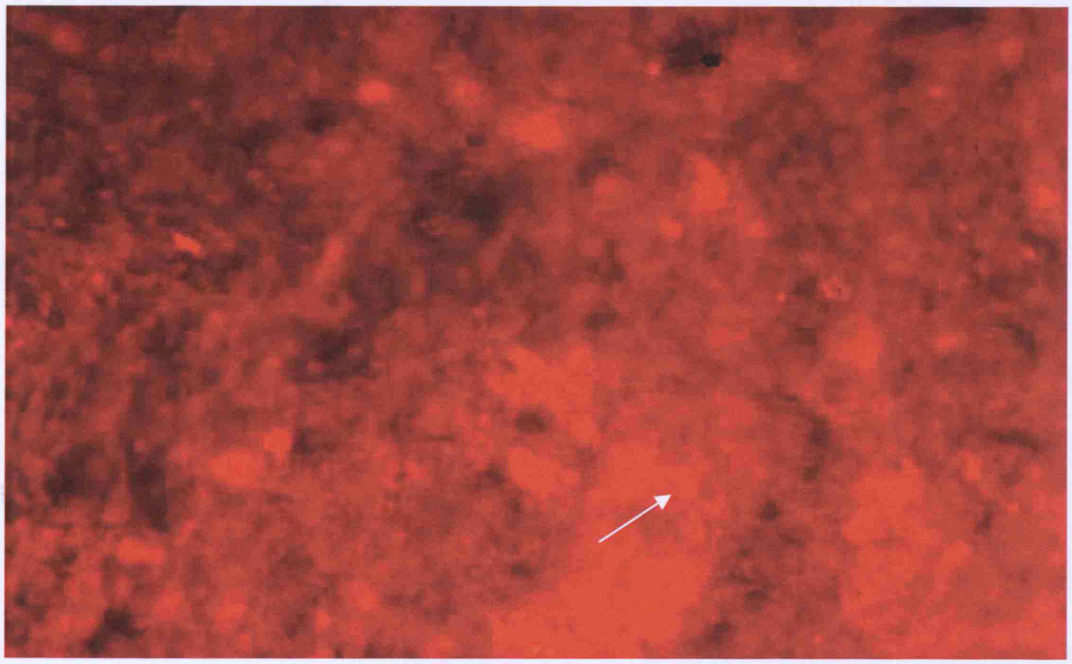


Figure 4.5 TUNEL assay showing a very few scattered apoptotic cells in tumours of Control M (A) and Control P (B) groups of the early gene transfer (×200), in which animals received either control plasmid or just PBS on day 0, along with colon cancer cells. Apoptotic cells are seen as red immunofluorescence (arrow).

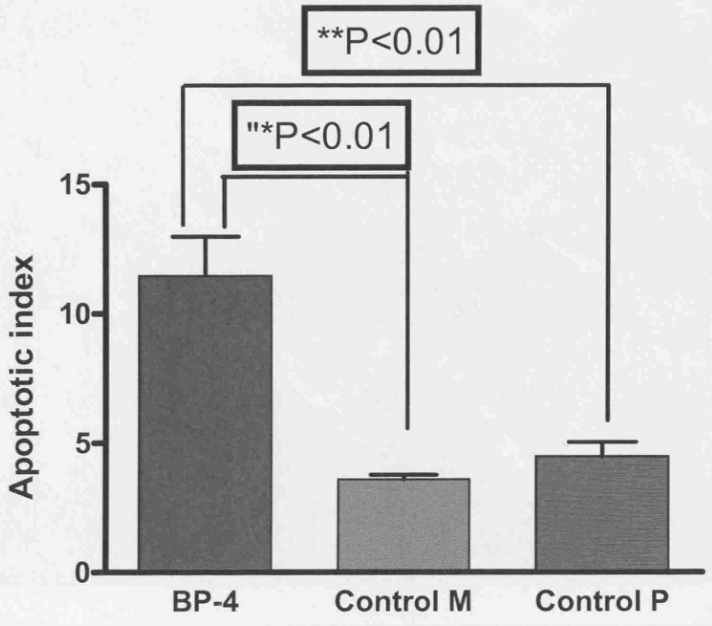


Figure 4.6 Comparison of apoptotic indices of early gene transfer model tumours among the three groups in which animals received gene construct with or without IGFBP-4 or none. BP-4 group tumours showed a higher apoptotic index than control groups. Values are shown as mean±SEM of 6 animals in each group. Standard error bar is shown. There was a statistical significant difference in the index between the BP-4 treated tumours and the two control group tumours.

4.3.2 Apoptosis assessment by electron microscopy

Electron microscopy was performed only for the preventive (early gene transfer) model. Apoptotic cells were confirmed by scanning the tumour sections with transmission electron microscopy (Figures 4.7 to 4.11). This method showed a higher numbers of cells undergoing apoptosis in BP-4 group tumours, while a less numbers of such cells were noted in the control group tumours.

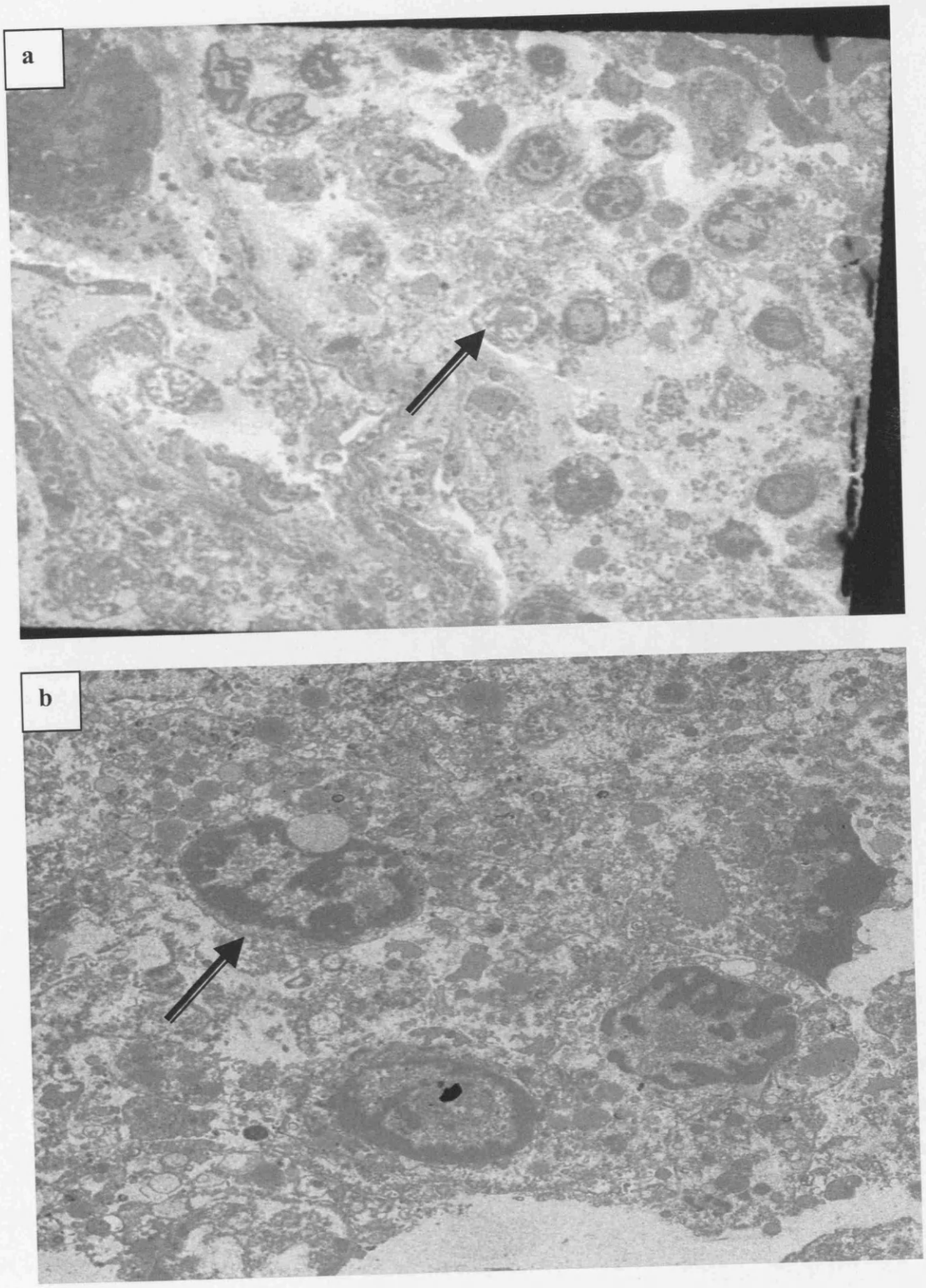


Figure 4.7 Transmission electron microscopic imaging a) low power (x710) b) high power (×11500) showing apoptotic cells which are seen as apoptotic bodies (arrow) with shrunken nucleus and condensed cytoplasm in BP-4 treated subcutaneous tumours of the early gene transfer model, in which animals received gene construct containing IGFBP-4 on day 0, at the same time as tumour induction.

Features typical of apoptosis include compaction and segregation of the condensed chromatin, that adjoins the inner surface of the nuclear membrane, condensation of the cytoplasm, and in the late stage of apoptosis chromatin fragments will be highly condensed.

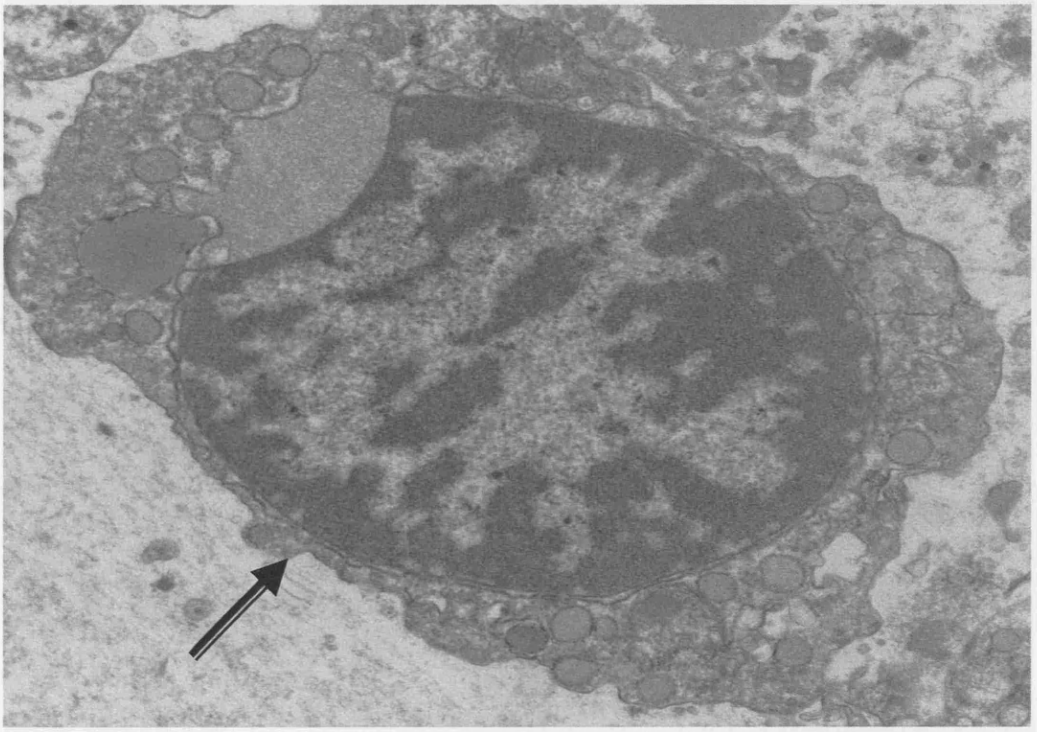
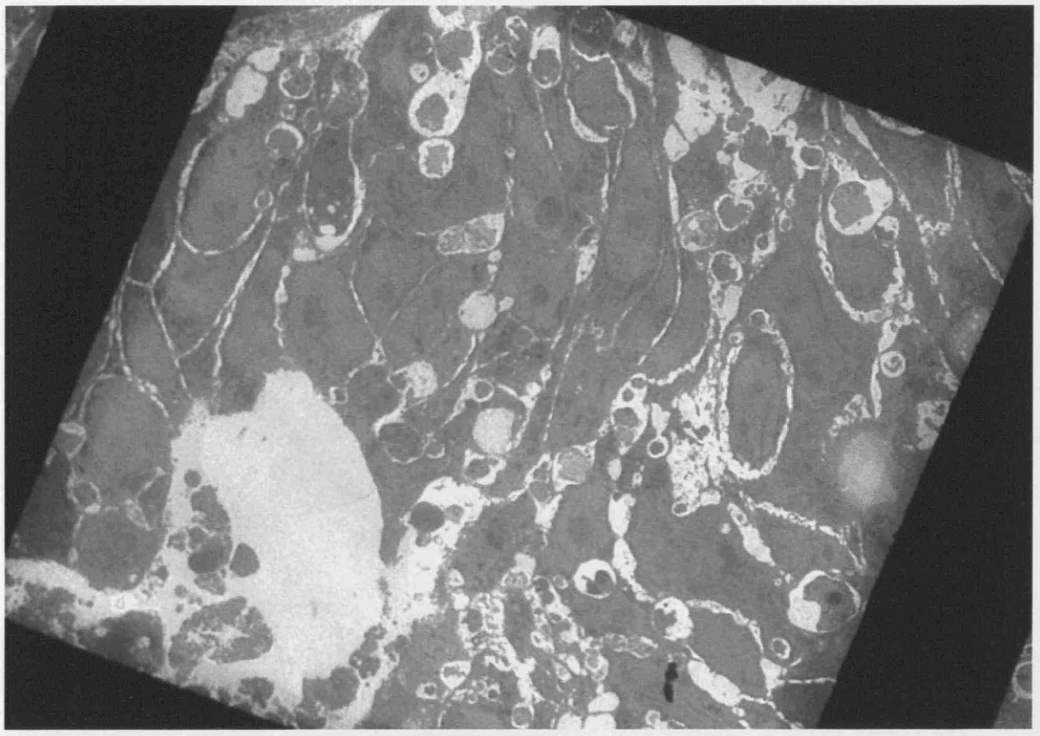


Fig 4.8 Electron microscopy (×14400) showing apoptotic cells with dense masses of chromatin, against nuclear membrane in BP-4 group tumours of early gene transfer model in which nude mice received one dose of gene construct containing BP-4 at the same time as tumour induction.

a



b

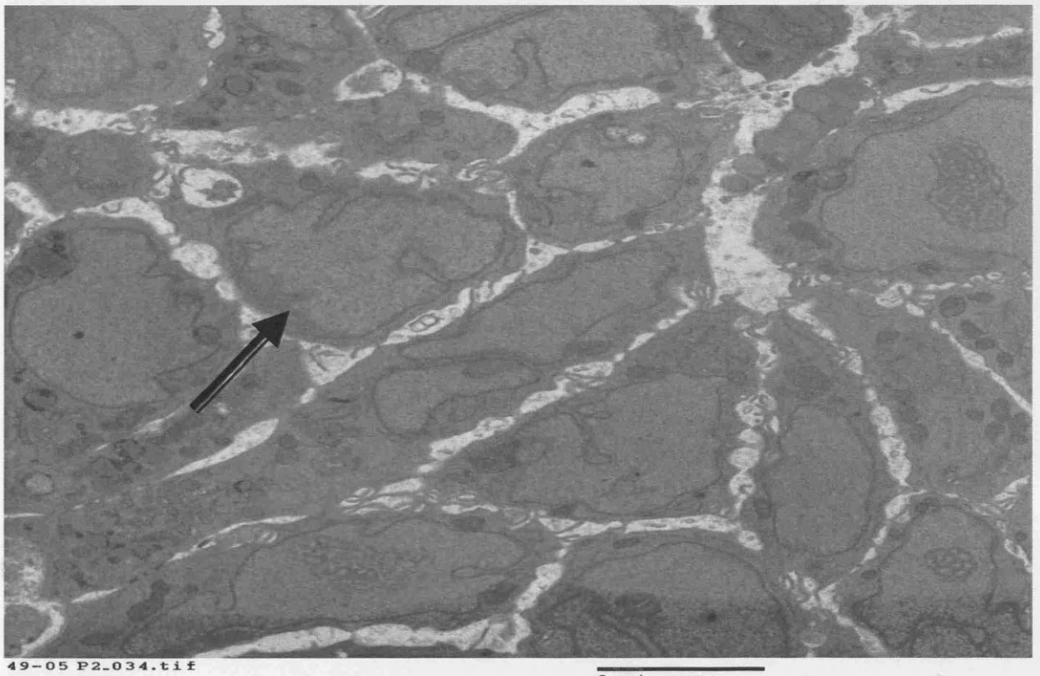


Figure 4.9 Electron microscopy (a) low power (X 710) (b) high power (×11500) showing colon cancer cells and necrotic cell. These figures belong to control P group tumours of the early gene transfer model, in which animals received animals received no gene therapy.

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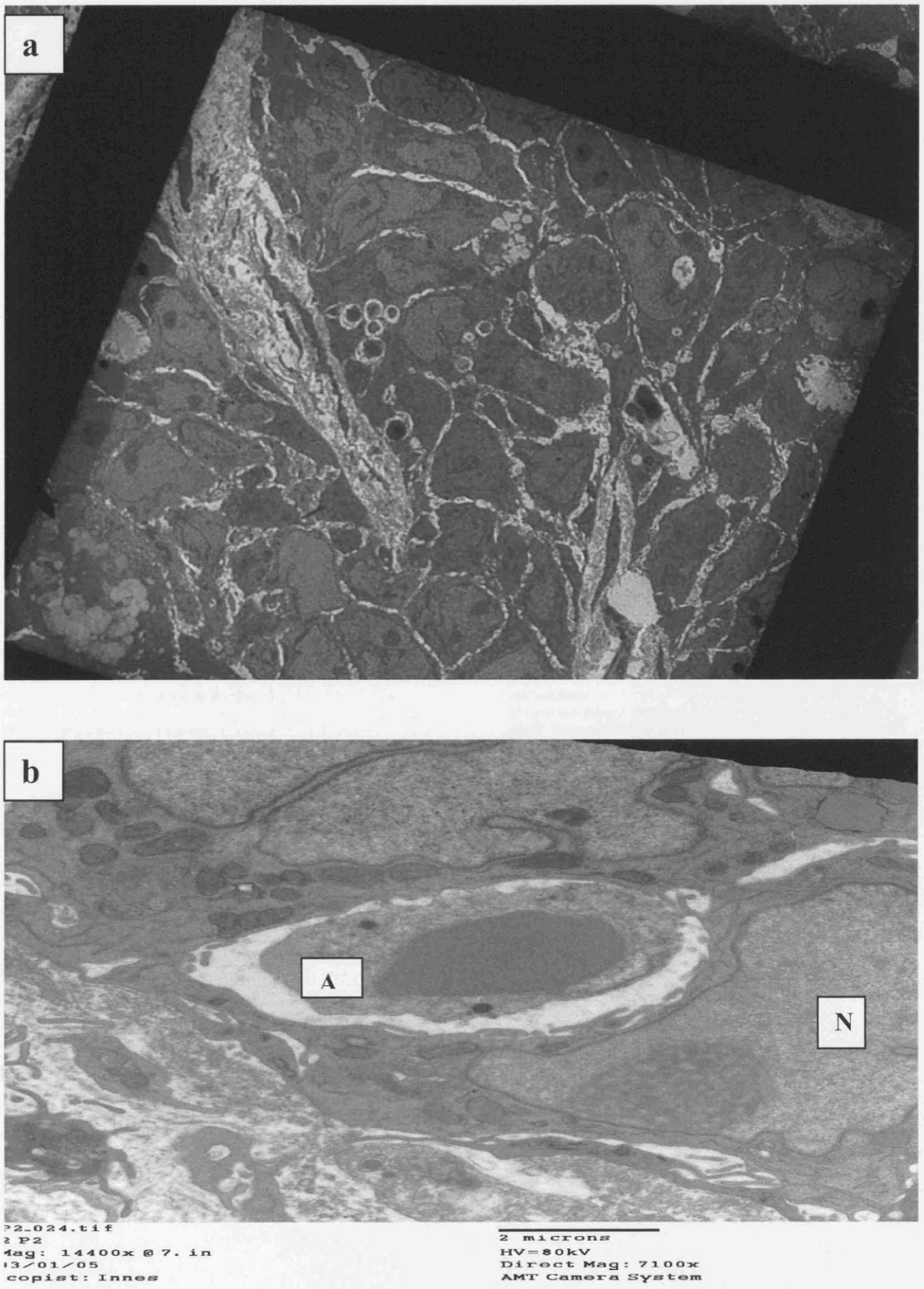


Figure 4.10 Electron microscopic images of control group M (a) (x 710) (b) (x14400) tumours of the early gene transfer model in which the animals received one dose of control plasmid on day '0' at the same time as subcutaneous tumour induction in nude mice. These figures show both necrosis and apoptosis. In figure b, an apoptotic cell is seen in the centre of the field with a condensed nucleus (arrow) (Key: N = Necrosis A = Apoptosis)

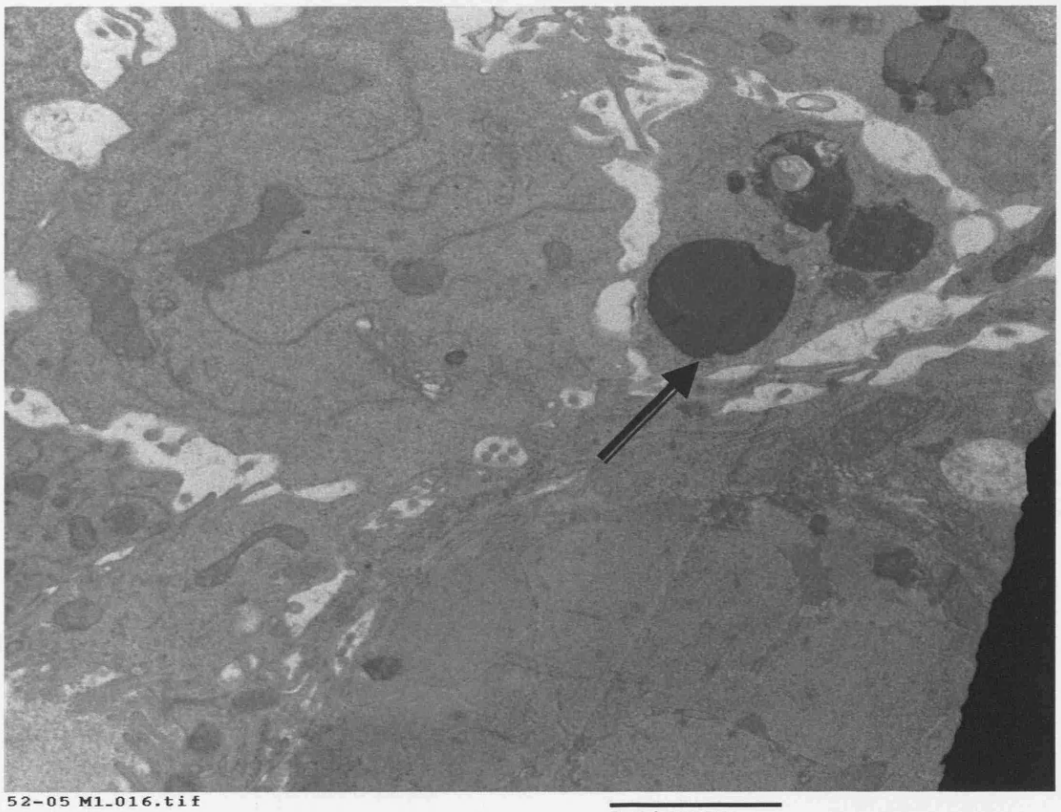


Figure 4.11 Electron microscopic image (x7100) showing both necrotic and apoptotic cells in Control M group tumours of the early gene transfer model, which were treated with one injection of control plasmid. Apoptosis is recognised by a condensed dark nucleus (arrow).

4.3.3 Effect of IGFBP-4 gene transfer on Bax and Bcl-2 proteins

In the late gene transfer model, Western blot densitometry analysis showed that the IGFBP-4 gene therapy significantly increased the expression of Bax (8.58 ± 1.55, 5.18 ± 0.37, BP-4 vs Control, P<0.05) (figure 4.13)and decreased the expression of Bcl-2 (10.81 ± 1.30, 12.27 ± 2.91, BP-4 vs Control, P=Not significant) in tumour tissues (figure 4.12).

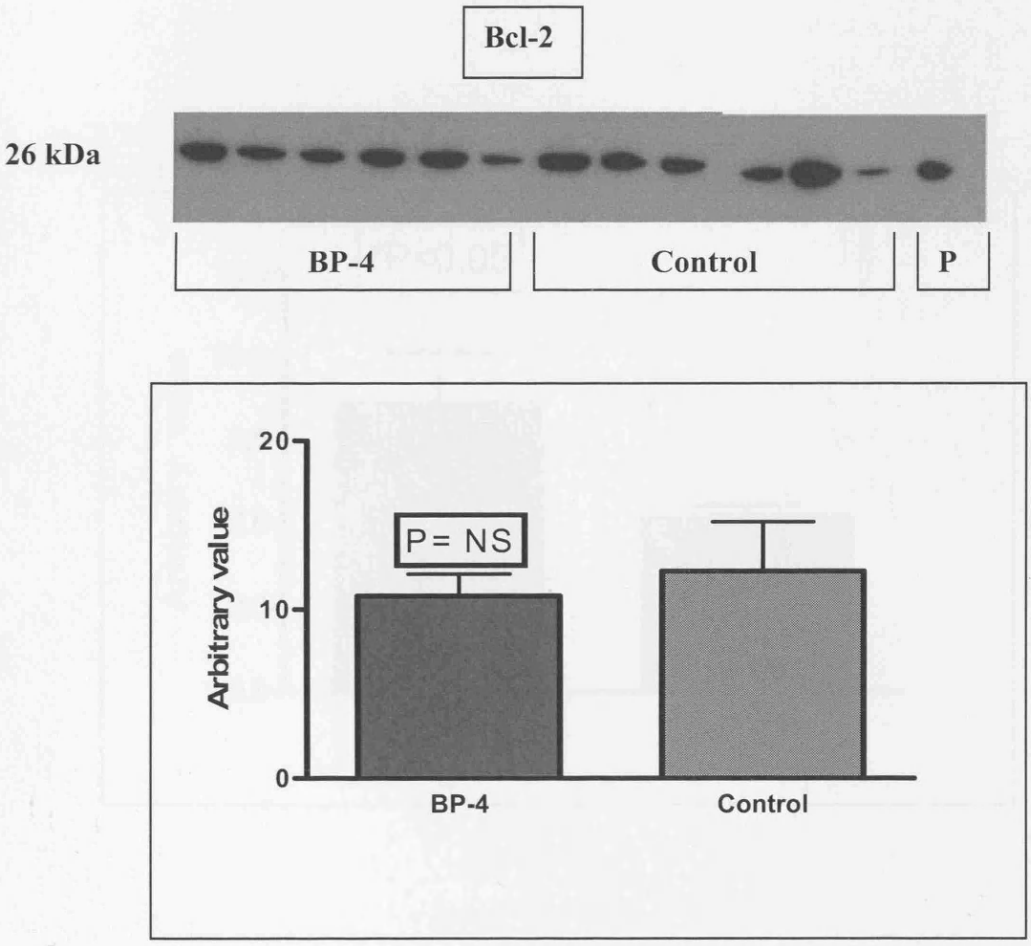


Figure 4.12 Western Blot and densitometry analysis of tumours of the late gene transfer model showing lower Bcl-2 expression by BP-4 group than control. Animals received one peritumoural injection of gene construct with or without IGFBP-4 on day 8, following induction of subcutaneous cancer. The values were shown as mean±SEM of 6 animals in each group. *(Key: B1- B6 represents BP-4 group and 1–M6 represents control group. P represents tumours without any treatment. NS = non significant).*

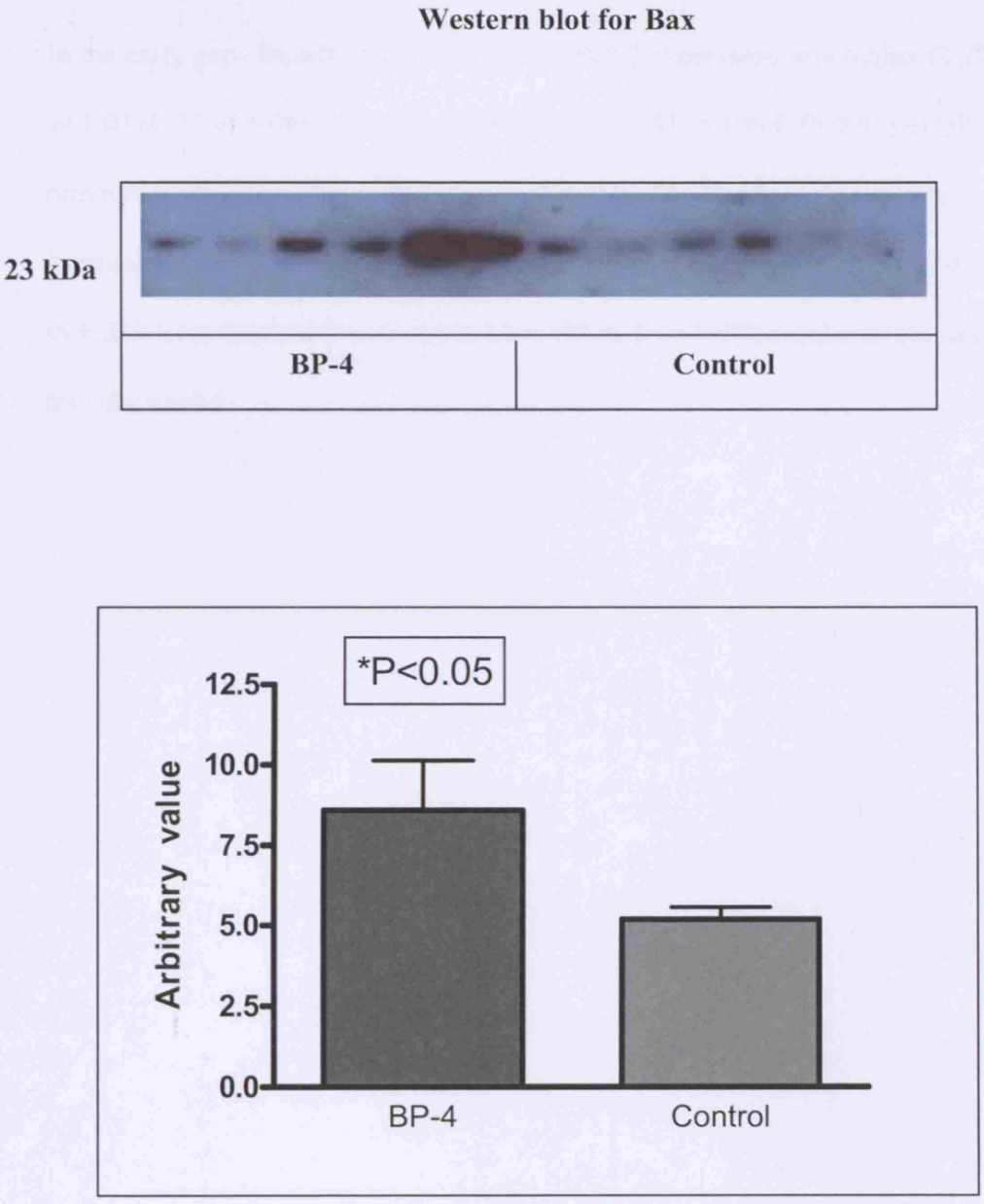
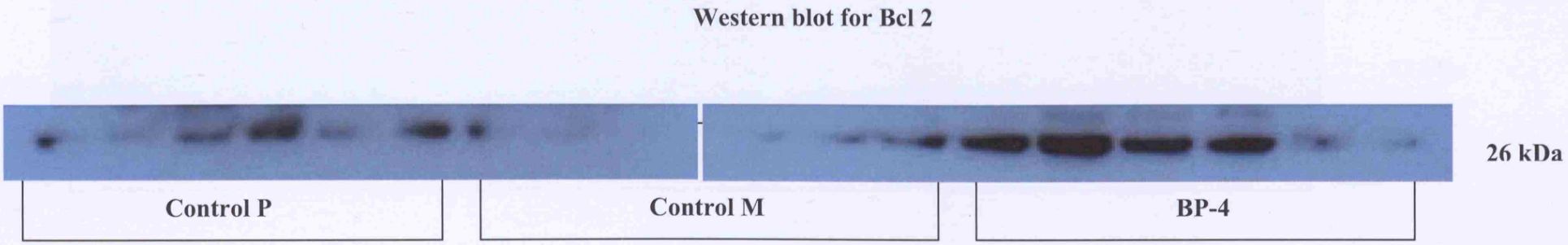


Figure 4.13 Western Blot and densitometry analysis of tumours of the late gene transfer model for Bax protein, showing significantly higher expression by BP-4 group than control. In this model, animals received one peritumoral injection of gene construct with or without IGFBP-4 on day 8, following induction of subcutaneous colon cancer. Results were expressed as mean±SEM of 6 animals per group.

Early gene transfer (Preventive) model

In the early gene transfer model, the mean Bcl-2 expression was higher (2.27±0.65 vs 1.01±0.25 vs 6.06±1.77, Control P vs Control M vs BP-4, $P<0.01$) in BP-4 treated tumours when compared with the control group. The Bax protein expression was also increased in BP-4 treated tumours after early gene therapy (3.37±1.15 vs 4.11±1.30 vs 6.31±1.16; Control P vs Control M vs BP-4; $P=0.2357$) similar to the late gene transfer model.



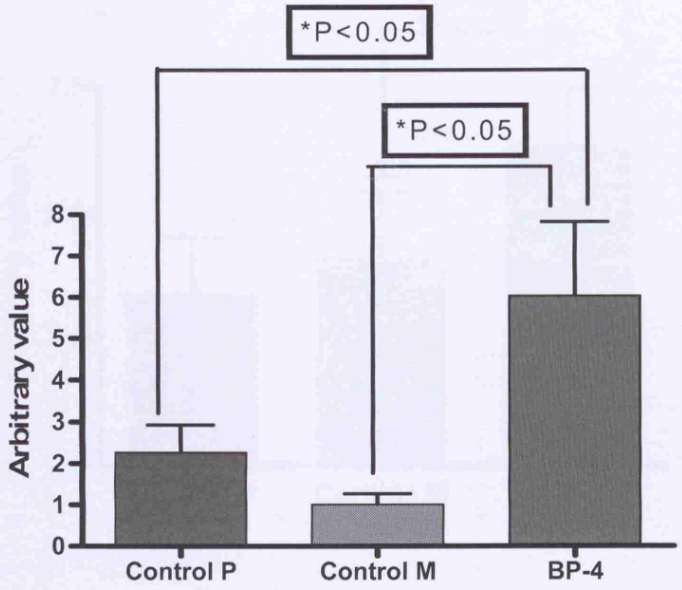
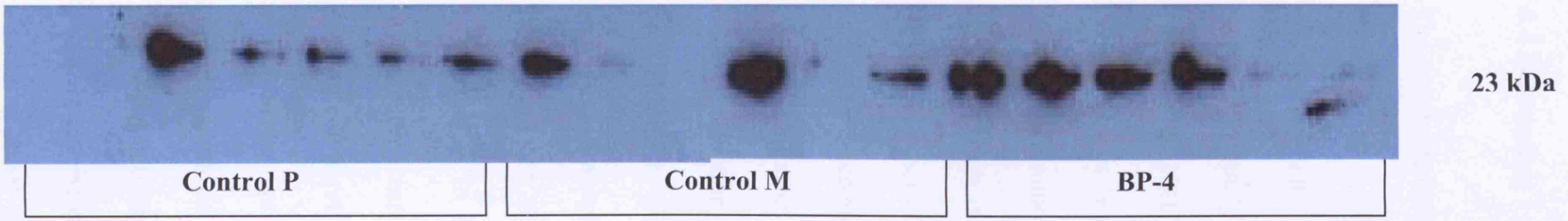


Figure 4.14 Western blot and densitometry analysis of tumours of early gene transfer model in which the animals received gene construct with or without IGFBP-4 or none, on day 0 at the same time as colon cancer induction, showed a higher expression of Bcl-2 by BP-4 group compared to Control P and M. Values are shown as mean ±SEM of 6 animals in each group.



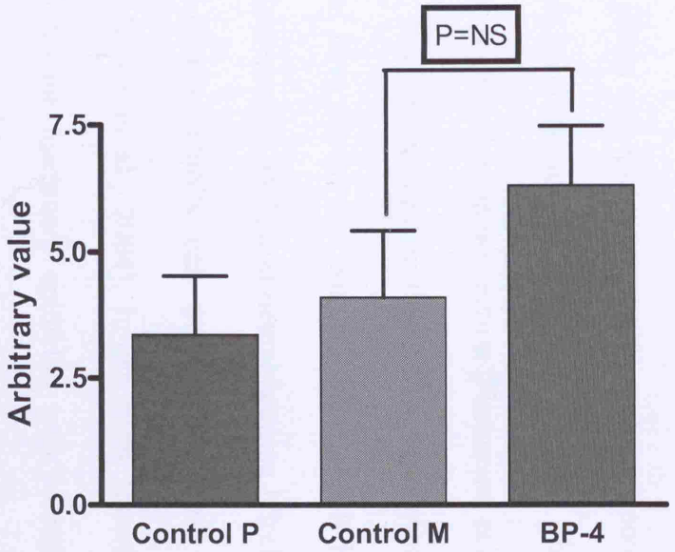


Figure 4.15 Western blot (a) and analysis by densitometry software (b) of subcutaneous tumours of early gene transfer model, in which animals received either plasmid with or without IGFBP-4 gene or none on day 0, at the same time when colon cancer was induced. BP-4 group tumours showed a higher expression of Bax protein when compared with control groups P and M. Values are shown as mean±SEM of 6 animals in each group. (*NS=not significant)*

4.4 Discussion

The word 'Apoptosis' is derived from the Greek language meaning shredding of leaves. It is a form of programmed cell death that eliminates compromised or superfluous cells and is distinct from accidental cell death (necrosis). Apoptosis involves an autolytic cascade in the target cell with DNA fragmentation preceding cell lysis. TUNEL assay provides a reliable method to stain and visualise apoptotic cells. Previous *in vitro* experiments have shown that IGFBP-4 inhibits proliferating and growth-promoting actions of IGFs in both malignant and normal cells [Zhou et al., 2004;Singh et al., 1996;Singh et al., 1994a]. Apoptosis is an active energy-dependent process [Chen and Istfan, 2000]. It is observed in several conditions such as hormone-dependent involution of prostate and endometrium, certain viral illness, parenchymal atrophy after duct obstruction and in cancers after chemotherapy and ionizing radiation [Yu et al., 2004;Stanley Robbins et al., 1995]. During apoptosis cells shrink in their size and there is condensation of chromatin resulting in the formation of apoptotic bodies [Stanley Robbins et al., 1995]. It is characterised by extensive DNA fragmentation [Rojo and Gonzalez, 1998] with no accompanying inflammation. In contrast to this, necrosis results in cellular swelling and there is inflammatory response. IGFs, by their anti-apoptotic action [Nickerson et al., 1997], prolong cell survival. In theory, any binding protein that reduces the levels of IGFs should favour apoptosis. Studies have shown that both IGFBP-3 and IGFBP-6 can mediate apoptosis in Caco-2 [Saksena M, 2002] and neuroblastoma [Grellier et al., 2002] respectively. IGFBP-4 excess inhibits cell proliferation and stimulates apoptosis in lymphoid tissues [Zhou et al., 2004]. It has been shown that the apoptotic effect of TGF-β1 in bovine mammary epithelial cells is mediated by

IGFBP-4 [Gajewska and Motyl, 2004;Zhou et al., 2004]. It has been suggested that IGFBP-4 may act by altering Bcl-2 and Bax levels [Chen and Istfan, 2001;Yu et al., 2004]. Mitochondria play an important role in the regulation of cell death. Bcl-2 family anti-apoptotic proteins are located in the outer mitochondrial membrane and promote cell survival. Mitochondria also have the ability to promote apoptosis by releasing cytochrome c, which in turn can activate caspase-9. Caspases are cysteine proteases that exist within the cell as inactive pro-forms or zymogens. These zymogens can be cleaved to form active enzymes during apoptosis. Caspase-3 and caspase-6 are responsible for the cleavage of the key cellular proteins that leads to the typical morphological changes that occur inside the cells during apoptosis.

Evidence has shown that IGFBP-4 increases apoptosis in prostate cancer cells in response to induction by 6-hydroxyurea compared with the controls [Damon et al., 1998]. The present study, for the first time, presents an evidence that the IGFBP-4 increases apoptosis in colon cancer cells, which was confirmed by TUNEL staining and electron microscopy. The increase in apoptosis induced by IGFBP-4 in colon cancer is also in line with the studies on prostate and ovarian cancers, both of which suggested that the IGFBP-4 played a role as a signal for apoptosis and it was overexpressed in involuting prostate by mediating apoptosis [Thomas et al., 2000;Bruyninx et al., 2000]. Although the actual mechanism of apoptosis remains to be explored, it can be assumed that it is either the local binding of IGF or an IGF-independent mechanism that mediates apoptosis. Experiments show that IGF-I protects HT-29 cells from apoptosis [Remacle-Bonnet et al., 2000] and blockade of IGF-IR increases apoptosis in the same cells [Reinmuth et al., 2002b]. In this study, the average numbers of mitotic figures, which reflect the proliferative action of IGFs, were significantly decreased after IGFBP-4 gene therapy. These provide indirect

evidences for an IGF-dependent apoptotic and anti-mitotic actions of IGFBP-4. Although it is evident that cell death could occur in the centre of any fast growing tumour due to relative hypoxia, here in BP-4 treated animals the cell death was also found in the periphery of the tumour.

Results of Western blot of our experiment showed that there was an increase in the expression of Bax protein in IGFBP-4 treated tumours when compared to the control group in both early and late gene transfer models. However, Bcl-2 was decreased in BP-4 treated tumours only in the late gene transfer group. The early gene transfer group tumours did not show such a decrease after BP-4 gene therapy. Our hypothesis of the mechanism of IGFBP-4 affecting the apoptosis in tumour is summarised in figure 4.16. The IGFBP-4 acts possibly via IGF-1R and influences Bax and possibly Bcl-2 proteins. In the cancer cells, Bax and Bcl-2 proteins act on caspases which trigger the apoptosis. Bax and Bcl-2 proteins show structural similarities with pore-forming proteins [Desagher and Martinou, 2000]. It has been suggested that Bax can form a transmembrane pore across the outer mitochondrial membrane, leading to loss of membrane potential and efflux of cytochrome c and the apoptosis inducing factor. It is thought that Bcl-2 can prevent this pore formation [Desagher and Martinou, 2000]. The hallmark of apoptosis is the cleavage of chromosomal DNA into nucleosomal units which is mediated by an enzyme caspase activated DNase. Normally, when IGF-I binds to IGF-IR, it activates phosphoinositide 3-kinase (PI3K), promotes cell proliferation by activating the protein kinase, and blocks apoptosis by inducing the phosphorylation and inhibition of proapoptotic proteins [Galvan et al., 2003]. This mechanism is possibly inhibited by IGFBP-4 which reduces the local tissue availability of free IGF-I. IGF-IR may influence apoptosis by

acting on E-cadherin, a epithelial adhesion molecule [Mauro and Surmacz, 2004]. Evidence show that down-regulation of E-cadherin is associated with the dedifferentiation, progression, and metastasis of colorectal cancer [Dorudi et al., 1993].

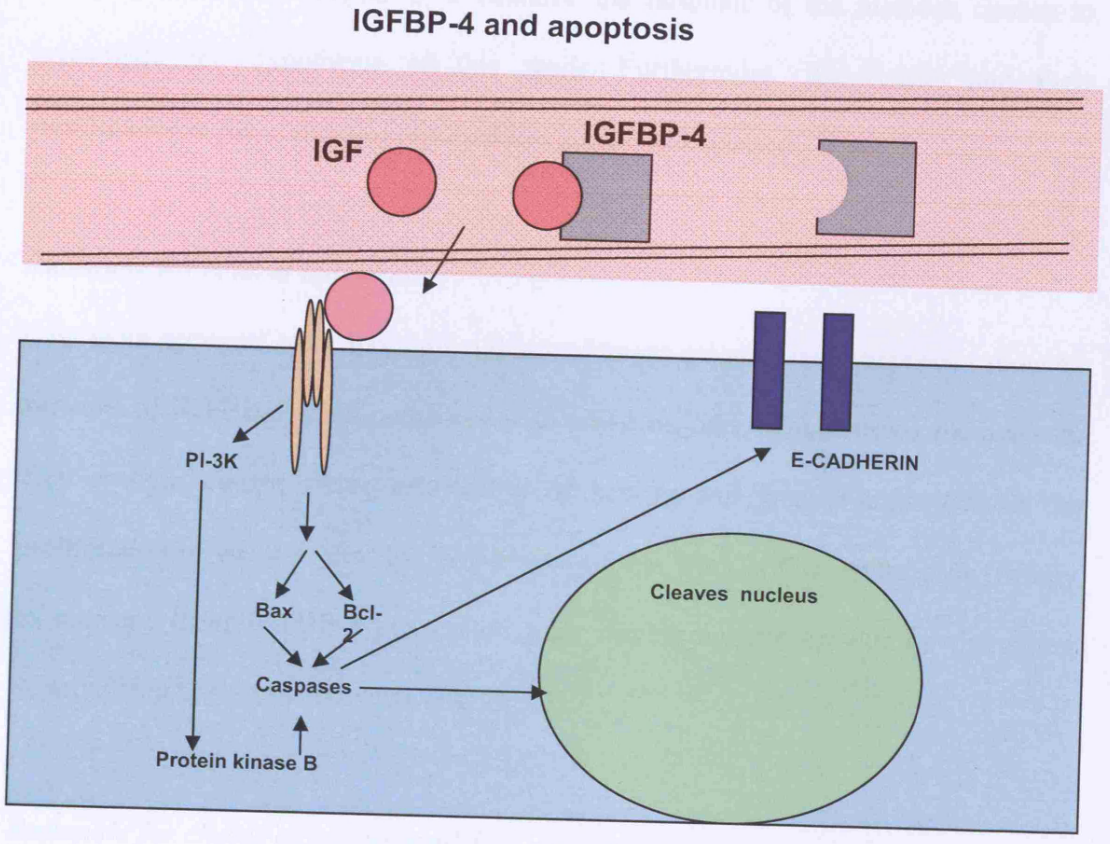


Figure 4.16 The possible molecular mechanisms of apoptosis mediated by IGFBP-4. The IGFBP-4 binds IGF-I and decreases its bioavailability to IGF-IR which in turn influences the levels of Bax and Bcl-2 proteins. The balance between these proteins is ultimately responsible for caspase mediated apoptosis.

This study showed that local treatment of IGFBP-4 gene therapy resulted in an increase in apoptosis of colon cancer *in vivo*. There was an associated increase in Bax protein suggesting that it could be the mechanism of apoptosis.

Chapter 5 General discussion and summary

The purpose of this chapter is to examine the rationale of the methods chosen to investigate the hypothesis of this work. Furthermore, the results and their significance in the context of reported literature are discussed.

Rationale for central hypothesis

Data from previous work has shown that malignant colonic tissue expresses reduced amounts of IGFBP-4 when compared with adjacent, non malignant colonic mucosa. This protein reduces tissue availability of key growth factors implicated in the proliferation of cancer cells. The hypothesis of this work is that, using gene therapy to increase local IGFBP-4 expression may inhibit tumour growth by disrupting positive feed back growth cycles (figure 5.3).

Rationale for choice of cancer model

A subcutaneous nude mouse cancer model was chosen over an orthotopic colon cancer model as the former allows for easier surveillance of tumour development and response. HT-29 cells were chosen because they are widely used, readily available and easy to culture.

Rationale for choice of gene therapy over administration of free IGFBP-4 protein or anti-IGF-IR

Arguably, IGF-1R activitiy could be curtailed by administration of either free IGFBP-4 or Anti-IGF-IR. In the first case, free IGFBP-4 would bind IGFs and reduce the amount of growth factor available to the IGF-1R. Anti-IGF-IR is a monoclonal antibody that binds to IGF-IR. Administration of this antibody would similarly reduce the amount of IGF-IR available to IGFs to bind to.

However, use of products which require systemic administration is dis-advantageous in a number of respects. Firstly, serum proteases are known to degrade free IGFBP-4 protein *in vivo,* thereby reducing half-life and bio-availability. Furthermore, delivery of the protein is difficult: daily injections around the tumour site are impractical; technology allowing tissue delivery via an implanated mini osmotic pump [Fowlkes et al., 2006] has not been used in humans to date. Commercially available IGFB-4 protein is considerably more costly than native expressed protein produced by gene transfer, and anti-IGF-1R is costly too. Finally, a proportion of the delivered protein or antibody will be taken up systemically and cause unwanted, non-localised inhibition of IGF-IR with associated untoward effect.

The ability to deliver plasmid containing IGFBP-4 cDNA via the method used in this work – i.e. single peri-tumoral injection – has the potential to allow clinical treatment of colon cancers accessed during colonoscopy or sigmoidoscopy.

Rationale for non-viral (plasmid) vector over viral vector

Viral vectors have a better gene transfer rate but they may have unwanted consequences as they are prone to host-mediated immune destruction once recognised as non-self. Plasmid mediated gene therapy is associated with less antigrnic stimulus [Lechardeur et al., 2005].Viral vectors are inefficient at transferring big transgenes [Reid et al., 2001;Kreiss et al., 1999] and may mutate. Plasmids are not associated with these problems, but do not self replicate except during normal cell division.

Rationale for development of two models –early and late

The first model (late gene transfer) was performed to assess the therapeutic effect of IGFBP-4. This effect will be useful in clinical settings such as locally advanced cancers, in which a patient needs palliation. The early gene transfer (Preventive) model may provide insight into the role of IGFBP-4 in the early stage of cancer establishment and growth. Comparison of both models may provide a better understanding of the role of IGFBP-4 in colon cancer *in vivo* rather than the single gene transfer model.

Plasmid therapy -mechanisms and effects

After administration of plasmid a proportion is taken up by proliferating colon cancer cells. The fate of the remaining portion is unknown, but presumumably lymphatic capillary clearance occurs. Adjacent tissues, such as muscle, may also clear some of this load.

Possible mechanism of entry of plasmid DNA into cells include a) large membrane disruption, (b) small membrane pores, and (c) receptor-mediated endocytosis

[Budker et al., 2000]. Experiments show that the major route for plasmid DNA nuclear entry is by passive nuclear importation during mitosis when the nuclear membrane temporarily breaks down [Grosse et al., 2006]. Following endocytosis of plasmid DNA, a large fraction of the DNA is ingested by lysosomes; it is degradation of plasmid DNA in these endo-lysosomes that constitutes one of the major impediments to efficient gene transfer. Plasmid DNA that escapes the endo-lysosomal compartment encounters the diffusional and metabolic barriers of the cytoplasm, greatly reducing the number of intact plasmids that reach the nucleosol. Nuclear translocation of DNA requires either the disassembly of the nuclear envelope or active nuclear transport via the nuclear pore complex [Lechardeur et al., 2005] . Little is understood about the fate of plasmid DNA after it reaches the nucleus [Li and Huang, 2000].

There are two potential mechanisms by which IGFBP-4 DNA is transcripted. Firstly, the plasmid cDNA may integrate into the DNA of the proliferating colon cancer cell DNA and multiplies as the native cell DNA replicates with cell division. This DNA produces IGFBP-4 protein by the usual mechanisms of transcription and translation. The second way in which IGFBP-4 is produced is by direct transcription of the plasmid cDNA independent of the host DNA. Figure 5.1 shows the possible mechanism of multiplication IGFBP-4 cDNA. Whichever mechanism is responsible, plasmid uptake appears to be improved when large volumes of plasmid DNA are administered. Rapid injection of plasmid DNA in a large volume (1.5 to 3.0 ml) through the portal or tail vein can give rise to high level gene expression in mice, particularly in the liver [Liu et al., 1999]. Reporter gene technology has been used to quantify plasmid uptake and subsequent efficieny of cDNA transcription [Liu et al., 1999].

Similarities between human and mouse IGF system

IGF-I is synthesised as a prohormone in the liver consisting of chains A, B, C, D and E. The mature protein has no E chain. IGF-I is 100% identical in human, pig and sheep. Human IGF-I closely resembles that of rats and mice, however 3 aminoacids in rats and 4 aminoacids in mice are different [Daughaday and Rotwein, 1989]. The human IGFBP4 gene is located on chromosome 17 [Allander et al., 1993] and spans about 15·3 kb [Zazzi et al., 1998]. According to the mouse genome sequence determined so far, the mouse IGFBP-4 gene spans 11·3 kb on chromosome 11 (http://www.ncbi.nlm.nih.gov/LocusLink/LocRpt.cgi?l=16010). Human IGFBP-4 cDNA encodes a 258 amino acid (aa) residue precursor protein which will generate the 237 aa mature human IGFBP-4. The human IGFBP-4 shares approximately 90% amino acid sequence identity with both rats and mice IGFBP-4 (http://marvester.embl.de/marvester/P478/P47879.html).

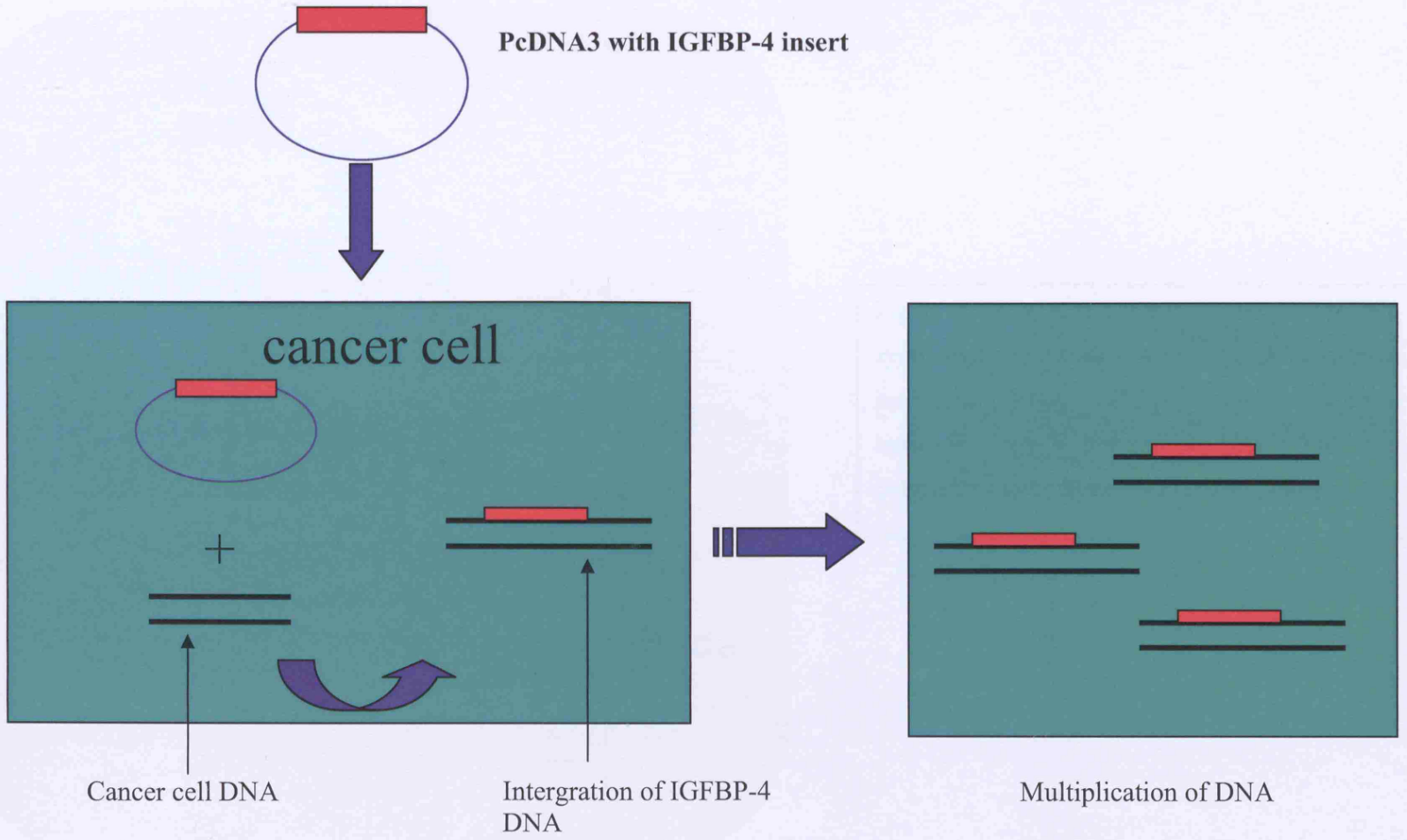


Figure 5.1 Showing proposed mechanism of integration and multiplication of IGFBP-4 DNA. The IGFBP-4 cDNA gets integrated with colon cancer cell DNA and multiplies along with them. Then it will result in increased mRNA levels which will be translated into IGFBP-4 protein.

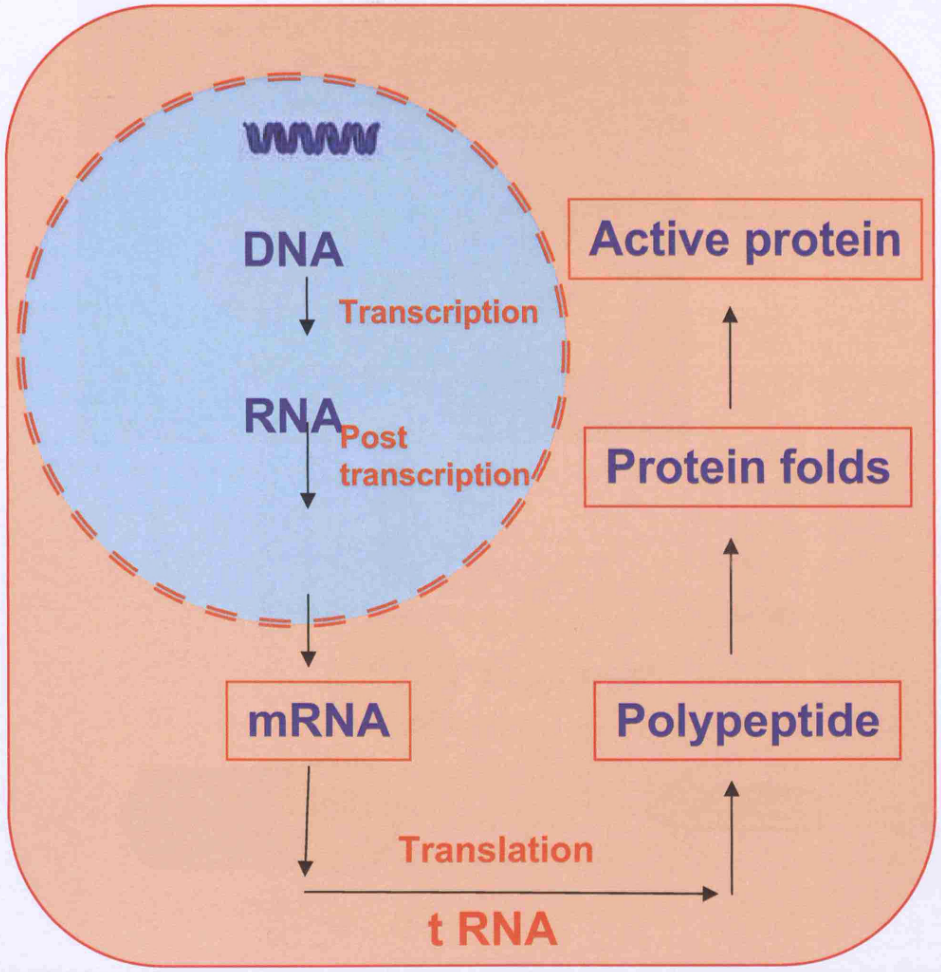


Figure 5.2 showing the cellular sequence of protein synthesis from IGFBP-4 gene. The DNA forms a messenger RNA (mRNA) which becomes translated into a polypeptide via tRNA. The polypeptide folds on itself to become protein.

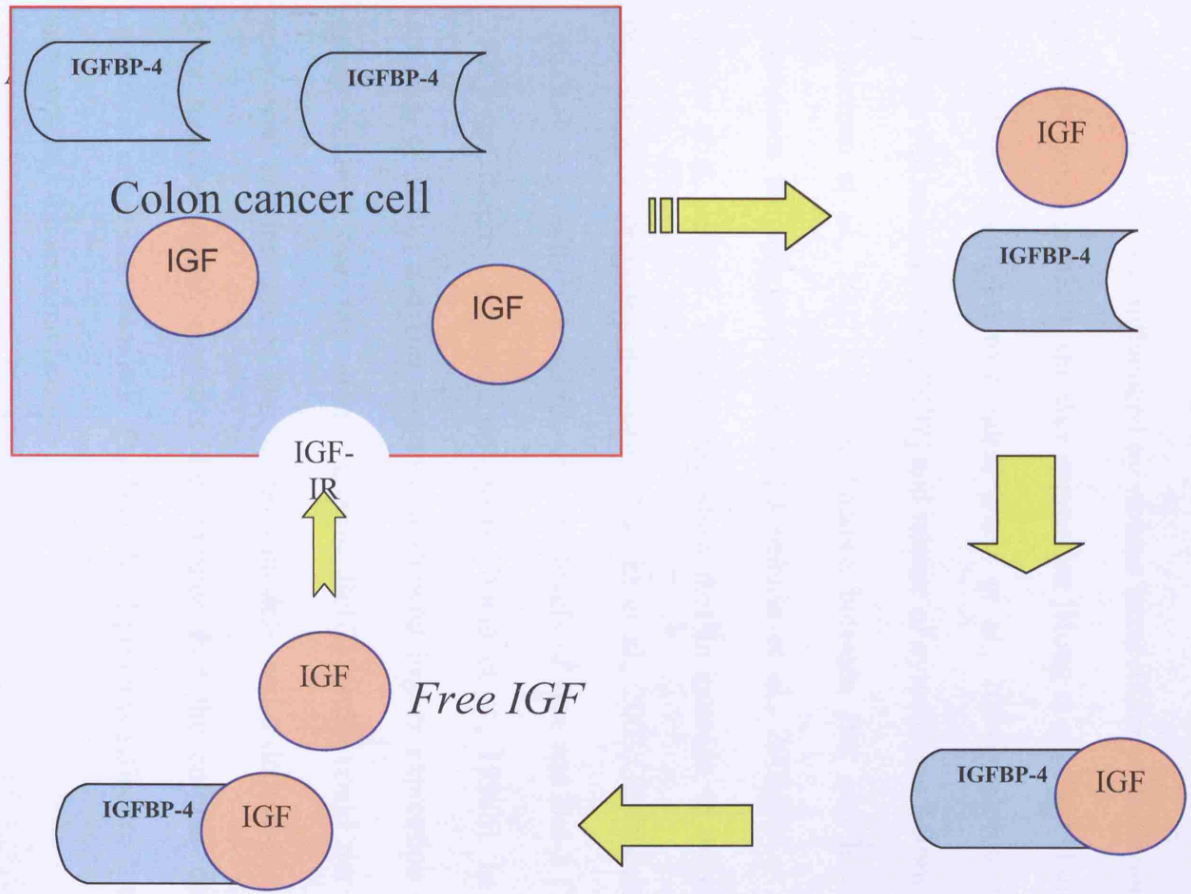


Figure 5.3 showing diagrammatic representation of feedback loop of IGFBP-4 acting on the cells which secrete them. The IGFBP-4 binds to IGFs and disrupts the feedback mechanism. IGF is required for cellular growth and proliferation. When there is less free IGF, cells can increase its sensitivity by increasing the numbers of IGF-IR expression.

The effect of IGFBP-4 gene transfer on apoptosis

Histopathological examination showed increased areas of cell death and fewer mitotic figures in tumours of BP-4 group when compared to control, in both early and late gene transfer models. TUNEL assay and electron microscopy both confirmed these dead cells as apoptotic cells. The apoptotic index of tumours of BP-4 group was significantly higher than control in both early and late gene transfer models. Apoptosis is influenced by various intracellular proteins and enzymes. IGF-I not only down-regulates the Bax expression [Hong et al., 2001] but also prevents its translocation to the mitochondria [Ness et al., 2004], inhibits the activation of caspase c [Linseman et al., 2002] and release of cytochrome 3 from the mitochondria [Linseman et al., 2002]. A fine balance between Bax and Bcl-2 proteins often determines the apoptotic process [Brambilla et al., 2003;Dai et al., 2000;Labat-Moleur et al., 1999]. Experiments show that an increase in the ratio of Bax/Bcl-2 proteins often precedes apoptosis [Bianchi et al., 2003;Giannakakou et al., 2001]. Insulin-like growth factors influence the levels of Bax and Bcl-2 [Yamamura et al., 2001] and thereby influence apoptosis [Wang et al., 1998b]. In our experiment, analysis of Bcl-2 and Bax expression showed higher expression of Bax by BP-4 group tumours than the control groups. Bcl-2 results could not be correlated to apoptosis. The increase in Bax by both models, and a decrease in Bcl-2 in the late gene transfer model, supports the findings that the cellular deaths are due to apoptosis rather than necrosis. The Bax/Bcl-2 proteins influence the caspases, which in turn affect the endonucleases.

Effect on IGFBP-4 expression

In the late gene transfer model, the IGFBP-4 expression was significantly higher in tumours of the BP-4 group when compared with the control group. However in the early gene transfer (Preventive) model, the expression of IGFBP-4 was higher in control P group tumours than the other two groups (BP-4 and control M). It indicates that the IGFBP-4 might have been used up by the tumour, or prior establishment of tumour may be a pre-requisite for IGFBP-4 expression. Another possible explanation is tumour cells, when deprived of IGF-I for a long time may be unable to produce IGFBP-4.

IGFBP-4 Protein formation from IGFBP-4 gene

Protein is formed inside the cell in two steps. The first step is the formation of mRNA from DNA (Transcription). The mRNA has 4 bases, but uracil will replace thymine of DNA. The next step is translation, in which the ribosomes read the mRNA and translate it into aminoacid sequence.

Before the synthesis of a protein begins, the corresponding RNA molecule is produced by RNA transcription. One strand of the DNA double helix is used as a template by the RNA polymerase to synthesize a messenger RNA (mRNA). This mRNA migrates from the nucleus to the cytoplasm. During this step, mRNA goes through different types of maturation including one called splicing when the non-coding sequences are eliminated. The coding mRNA sequence can be described as a unit of three nucleotides called a codon.

Protein translation involves the transfer of information from the mRNA into a peptide, composed of amino acids. This process is mediated by the ribosome, with the adaptation of the RNA sequence into amino acids mediated by transfer RNA. There are > 20 different tRNA molecules. Numerous initiation and elongation factors also play a role. Translation requires a lot of energy, with the hydrolysis of approximately 4 NTP→ NDP per amino acid added. Translation involves 3 processes: initiation, elongation, and termination.

The ribosome binds to the mRNA at the start codon (AUG) that is recognized only by the initiator tRNA. The ribosome proceeds to the elongation phase of protein synthesis. During this stage, complexes, composed of an amino acid linked to tRNA, sequentially bind to the appropriate codon in mRNA by forming complementary base pairs with the tRNA anti-codon. The ribosome moves from codon to codon along the mRNA. Amino acids are added one by one, translated into polypeptide sequences dictated by DNA and represented by mRNA. At the end, a release factor binds to the stop codon, terminating translation and releasing the complete polypeptide from the ribosome.

The events following biosynthesis include post-translational modification and protein folding. During and after synthesis, polypeptide chains often fold to assume, so called, native secondary and tertiary structures. This is known as protein folding.

Many proteins undergo post-translational modification. This may include the formation of disulfide bridges or attachment of any of a number of biochemical functional groups, such as acetate, phosphate, various lipids and carbohydrates.

Enzymes may also remove one or more amino acids from the leading (amino) end of the polypeptide chain, leaving a protein consisting of two polypeptide chains connected by disulfide bonds.

The IGF system and colorectal carcinogenesis

The IGF system itself is complex and it plays an important role in all stages of cancer formation. How and when the IGFs act on cancer cells in not clear. High blood levels of IGF-I is observed in patients with colon cancer [Tripkovic et al., 2007]. Low levels of circulating IGF-I retard the progression and metastatic potential of a number of cancers [Yakar et al., 2005]. IGF-II is an important regulator of tumour growth, both in early adenoma and in the progression to carcinoma [Christofori et al., 1994;Hassan and Howell, 2000]. Loss of IGF-II imprinting has been shown to be associated with the risk of developing colon cancer [Liou et al., 2007;Cruz-Correa et al., 2004]. The expression level of IGF-II was significantly higher in colorectal cancer than in normal colorectal mucosa [Xu et al., 1999]. IGF-IR is crucial for tumour transformation and survival of malignant cell, but is only partially involved in normal cell growth [Larsson et al., 2007]. At present there is not enough evidence available to prove that IGFs alone cause or initiate cancer.

Our experiments showed that IGFBP-4 gene therapy in the form of local gene therapy caused increased apoptosis. It did not prevent the cancer formation. The long term outcome of this gene therapy was unknown. Bax protein was increased in both early and late gene transfer models. It appears that the IGFBP-4 does not inhibit tumour growth *in vivo*. The results are summarised in figure 5.4.

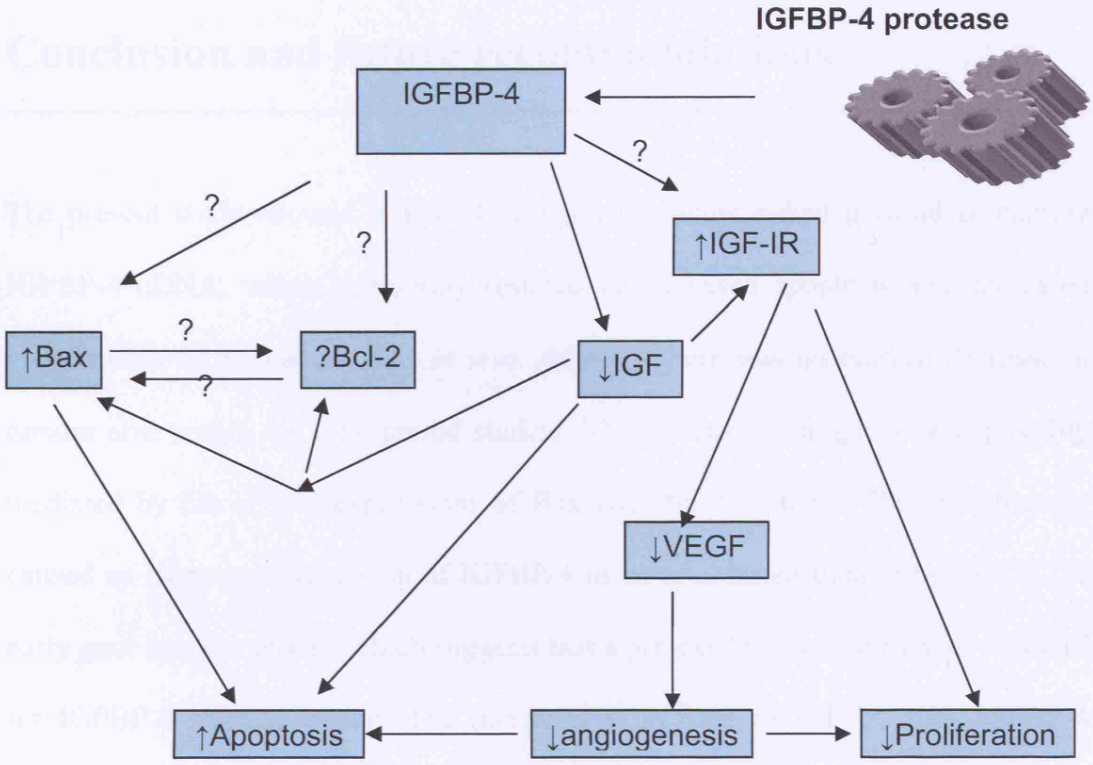


Figure 5.4 showing overall results of both experiments. IGFBP-4 gene therapy resulted in increased Bax and IGF-1R levels and these were associated with increased apoptosis and decreased mitosis. (Key: ?= mechanism to be studied)

Conclusion and future recommendations

The present study showed IGFBP-4 upregulation using naked plasmid containing IGFBP-4 cDNA, which apparently resulted in increased apoptosis and decreased proliferation of the cancer cells *in vivo*, although there was no marked decrease in tumour size within the time period studied. The increase in apoptosis was possibly mediated by the altered expressions of Bax and Bcl-2 proteins. The gene therapy caused an increased expression of IGFBP-4 in an established tumour but not in the early gene transfer model, which suggests that a pre-existing tumour may be required for IGFBP-4 overexpression. The increased expression of IGF-IR after IGFBP-4 gene therapy may be due to a feedback mechanism to compensate for the decreased free IGF level. This study showed that IGFBP-4 had some therapeutic potential. However, further studies are required to determine the timescale that is needed for the tumour size to diminish, to find out at what stage the gene therapy should commence, and to assess whether IGFBP-4 gene therapy can be combined with a low dose chemotherapeutic agent to achieve a better therapeutic effect.

Appendix

Table A 1 Clinical, epidemiological and population based studies linking IGF system and colorectal cancer

Reference (First author, year)	**Study Type**	**Population**	**Cases/control**	**Parameters**	**Result /conclusion**
[Nomura et al., 2003]	NCC	9,345	282/282	Serum IGF-I and IGFBP-3	No significant relation between IGF-I, IGFBP-3 and CRC
[Giovannucci et al., 2000]		32,826	276/236	Plasma IGF-I and IGFBP-3	↑IGF-I and ↓IGFBP-3 are associated with ↑ risk of large or tubulovillous/villous colorectal adenoma and cancer
[Kaaks et al., 2000]	CC	14 275	102/200	C-peptide, IGF-I, and IGFBP-1, -2 and -3	CRC risk was not related to IGF-I but ↑ for highest quintile of IGFBP-3 (OR = 2.46, 95% CI = 1. 09-5.57)
[Ma et al., 2000]	NCC	14916	193/318	Plasma IGF-I, IGF-II and IGFBP-3	Circulating IGF-I and IGFBP-3 are related to future CRC but IGF-II is not related
[Palmqvist et al., 2003]	NCC	NA	168/336	Plasma IGFBP-1, IGFBP-2 and insulin	IGFBP-1 and IGFBP-2 showed no association while chronic hyperinsulinemia is moderately associated with CRC risk
[Probst-Hensch et al., 2001]	Cohort	18244	135/661	Serum IGF-I, IGF-II and IGFBP-3	Serum IGF-I was not associated with risk of CRC but ↑ circulating IGF-II and IGFBP-3 may indicate impending CRC
[Teramukai et al., 2002]	CC	803	157 /311	Plasma IGF-I and IGFBPs	Non-significant ↑ in the prevalence odds of colorectal adenomas for highest versus lowest quartile level of IGF-I
[Hunt et al., 2002]	NCC	14275	102/200	Serum IGF-II	Possible ↑ in CRC risk with ↑IGF-II
[Ma et al., 2001]	NCC	14916	193/318	Plasma IGF-I and IGFBP-3	Dairy products cause a modest ↑in IGF-I levels. Low- fat milk lowers the risk of CRC particularly among individual with high IGF-I/IGFBP-3

[Miraki-Moud et al., 2001]	CS	NA	81/26	Serum IGF-I, IGF-II, IGFBP-2 and-3	IGFBP-2 may regulate the bioavailability of IGF, which modulate colonic cell proliferation and or differentiation
[Renehan et al., 2001a]	Prospe Ctive	442	53/47	Serum IGF-I and IGFBP-3	IGF-I and IGFBP-3 levels are related to future CRC risk and may predict adenoma progression
[Renehan et al., 2000a]	Co	NA	52/293	IGF-I ,IGF-II, IGFBP-2 and -3	Serum IGFBP-2 may have an adjunct role in CRC surveillance (↓ in IGFBP-2 level post surgery)
[Jenkins et al., 2000]	CC	NA	66/0	Serum IGF-I	Development of new adenomas, but not hyperplastic polyps, was associated with ↑ serum IGF-I ($P < 0.005$)
[Manousos et al., 1999]	CCl	NA	41/50	IGF-I, IGF-II and IGFBP-3	Individuals with IGF-I and -II values in upper 2 tertiles had ↑ odds ratio for CRC (OR = 5.2, 95% CI 1.0-26.8) compared with those in lower tertile
[el Atiq et al., 1994]	Co	NA	17/0	IGFs and IGFBPs	Simultaneous elevation of serum IGFBP-2 &-3 in CRC
[Renehan et al., 2001b]	CCl	NA	60/306	Serum IGF-I, IGF-II IGFBP-2 and -3	IGF-II and IGFBP-2 ↑ in acromegalics but presence of colorectal neoplasia did not contribute to ↑ in serum IGF-II and IGFBP-2
[Renehan et al., 2000b]	CC	NA	92/57	Serum IGF-I, IGF-II, IGFBP-2 and -3	Significant association between adenoma occurrence, ↑ IGF-II ($P<0.0001$) and serum IGFBP-2 ($P<0.0001$). Removal of adenoma led to ↓ in IGF-II ($P<0.001$) and IGFBP-2 ($P<0.001$)
[Baciuchka et al., 1998]	Co	NA	13/0	IGFBP-3 and IGFBP-3 protease	Inhibition of IGFBP-3 proteolysis and invasion of colon cancer may be related

NA - Not applicable, CC - case control, NCC – nested case control, Co - comparative, CS-cross sectional

Table A2 *In vitro* and *in vivo* experimental studies linking the IGF system and colon cancer

Reference (First author, year)	**Study**	**Type**	**Cell line**	**Parameters**	**Result /Conclusion**
[Kansra et al., 2000]	Effect of TGF-β on IGFBP-3	*In vitro*	A variety of cell lines	Proliferation	TGF β ↑ IGFBP-3; antisense inhibition of IGFBP-3 blocks growth-promoting effect of TGF-β in all cell lines
[Culouscou and Shoyab, 1991]	Identification of a growth nhibitor from serum-free conditioned medium	*In vitro*	HT-29	Proliferation	Isolated growth inhibitor identical to human IGFBP-4
[Saksena M, 2002]	Effect of IGFBP-3 cDNA	*In vitro*	Caco-2	Apoptosis	Colony formation ↓ (50%) in IGFBP-3 transfected cells compared to control. IGF-I ↑ colony formation in both transfected and control cells
[Kim et al., 2002c]	Effect of antisense IGFBP-6 cDNA	*In vitro*	Caco-2	Proliferation	Antisense clones grew faster and the final density was 31 ± 3% higher than the control
[Leng et al., 2001a]	Influence of IGF-II on Butyrate and trichostatin A induced apoptosis	*In vitro*	LIM 2405	Cell growth and apoptosis	IGFBP-3 levels ↑ by butyrate and trichostatin and IGF-II inhibited apoptotic effects of both agents
[Singh et al., 1994a]	Effect of episomal expression of sense and antisense IGFBP-4 c DNA	*In vitro*	HT-29	Proliferation	Antisense cells showed ↑ basal and IGF-I-stimulated growth but Sense cells did not show any such increase, suggesting that IGFBP-4 overexpression was not inhibitory to HT-29 cells
[Zhang et al., 1995]	Expression of IGF-II and IGFBPs	*In vitro*	Caco-2	IGF-II, IGFBP and differentiation	Expression of IGF-II, IGFBP-2, and IGFBP-6 is regulated in a differentiation-dependent manner
[Oh et al., 2001]	Effect of 1α,25-(OH)(2)D(3) and Vitamin D analogues (EB1089, CB1093), 1β,25-(OH)(2)D(3)	*In vitro*	HT-29	Proliferation	All analogues except 1β ,25-(OH)(2)D(3) inhibited cell proliferation, but relative potencies of EB1089 and CB1093 were greater than that of native vitamin

[Kim et al., 2002b]	Effect of trans retinoic acid(Tra): role of IGFBP-6	*In vitro*	Caco-2	Proliferation	Dose dependent ↓ proliferation caused by Tra may be from IGFBP-6-mediated inhibition of IGF-II
[Ewton et al., 2002]	Role of IGF-I and IGF-II	*In vitro*	NIH-3T3	Differentiation and proliferation	IGF-I was initially mitogenic, and then caused growth arrest and differentiation. Concurrent IGFBP-3 addition blocked growth inhibition by IGF-I and IGF-II
[Kim et al., 2000]	Effect of poly unsaturated fatty acid	*In vitro*	Caco2	Proliferation, IGF-II and IGFBP-6	↓ proliferation of Caco-2 may be due to ↑ IGFBP-6 which binds to IGF-II
[Kim et al., 2002a]	Effect of Trans-10, cis-12-conjugated linoleic acid (CLA)	*In vitro*	Caco-2	Proliferation	CLA inhibits Caco-2 cell growth by↓IGF-II secretion
[Kirman et al., 2002]	Effect of plasma from patients undergoing major surgery	*In vitro*	HT-29	Proliferation	Major open surgery led to depletion of IGFBP-3 in blood that promote HT29 tumour cell proliferation *in vitro*
[Collard et al., 2003]	Role of IGFBP-3 in sodium butyrate(NaBt) induced apoptosis	*In vitro*	Adenoma cells	Apoptosis	IGFBP-3 may act as a positive regulator of NaBt-induced apoptosis by IGF independent mechanism
[Cho et al., 2003]	Effect of CLA isomers(trans-10, cis-12 conjugated linoleic acid)	*In vitro*	HT-29	Cell proliferation	t10c12 CLA ↓ IGF-II level in a dose-dependent manner, whereas c9t11 CLA had no effect
[Leng et al., 2001b]	Effect of IGFBP-6 and IGF-II	*In vitro*	LIM 1215	Proliferation and adhesion	IGFBP-6 inhibits IGF-II-induced but not basal proliferation and adhesion of LIM 1215 cells
[Mishra et al., 1998]	Assay of IGF-I receptor, IGF-I and IGFBP-2	*In vitro*	Cancer tissue	IGF-I , IGF-IR and IGFBP-2 mRNA	IGFBP-2 mRNA ↑ 4-8-fold in CRC than controls and was highest in Dukes C samples. IGFBP-2 mRNA was localized to malignant cells and not to stroma
[Michell et al., 1997b]	Expression of IGFs and IGFBPs	*In vitro*	Cancer tissue	mRNAs of IGFs and IGFBPs	Both IGF-I and IGFBP-2 mRNA were expressed by all normal and cancer samples but IGF-II mRNA was only detected in cancer tissue

[Remacle-Bonnet et al., 1997]	Effect of surface-bound plasmin	*In vitro*	HT29-D4	IGFBP-4 proteolysis	Proteolysis by plasmin can promote autocrine/ paracrine IGF-II bio-availability in colon-cancer cells
[Michell et al., 1997a]	Effect of IGF-I and Des(1,3)IGF-I	*In vitro*	COLO205 HT29 SW620	Cell sensitivity to IGF-I and IGFBPs	In all 3 cell lines, cell-conditioned media ↓ sensitivity to IGF-I but not to des(1,3)IGF-I suggesting that IGFBPs inhibit action of IGF-I
[Nishimura et al., 1998]	Effect of short-chain fatty acids	*In vitro*	Caco-2	IGFBP-2 and -3	Butyrate ↑ the secretion of IGFBP-2 in a dose-dependent and reversible manner and ↓ the secretion of IGFBP-3
[Akagi et al., 1998]	Effect of IGF-I ,Des-(1-3)-IGF-I	*In vitro*	HT29	VEGF mRNA	IGF-I ↑ the activity of VEGF promoter. IGF-I and Des-IGF-I had similar effects on VEGF mRNA
[Hoflich et al., 1998]	Effect of IGFBP-2 on colon cancer cells	*In vitro*	LS513, HT-29	Proliferation	IGFBP-2 inhibits proliferation in IGF-responsive colon carcinoma cell lines
[Perer et al., 2000]	IGF-I receptor antagonism in chemoradiation therapy	*In vitro*	SW 480 cells	Inhibition	IGF-IR antagonism ↑ the cytotoxic effect of chemo radiation therapy
[Di Popolo et al., 2000]	IGF-IR and COX-2 activity	*In vitro*	Caco-2	COX-2 and IGF-II mRNA and PGE-2	Antibody to IGF-IR inhibited COX-2 mRNA expression and dominant negative IGF-IR ↓ COX-2 expression and activity
[Williams et al., 2000]	Effect of IGFBP-3	*In vitro*	Colon cancer cells	IGFBP-3, apoptosis and differentiation	IGFBP-3 alone have no effect on growth of colon cancer cells but it enhances P-53 dependent apoptotic response to DNA damage
[Singh et al., 1994b]	Expression of IGF-II and IGFBPs by different colon cancer cell lines	*In vitro*	COLO 205, Caco-2, HCT 116, HT-29	mRNA and protein of IGFs and IGFBPs	All cell lines expressed IGFBP2 and/or IGFBP4 mRNA and secreted IGFBP4 and/or IGFBP2; IGFBP1 was not detected in any cell line. IGFBP3 mRNA was detected only in IGF responsive cellS
[Freier et al., 1999]	Expression of the IGFs and their receptors	*In vitro*	Cancer tissue	IGF-I ,II and IGF-IR	IGF-I polypeptide, not mRNA, was present in small amounts in normal and malignant tissue. IGF-II was expressed 40 times IGF-IR 2.5 times and IGF-IIR 4 times more in colonic tumours

[Reinmuth et al., 2002a]	Transfection with a truncated dominant-negative form of IGF-IR (IGF-IRDN).	*In vitro* & *in vivo*	KM12L4 nude mouse	VEGF expression	IGF-IRDN cells showed ↓ level of VEGF mRNA and protein. In nude mouse it led to ↓ tumour growth,VEGF expression,& vessel count ($p < .05$)
[Reinmuth et al., 2002	Blockade of IGF-IR and angiogenesis	*In vitro* & *in vivo*	HT-29 nude mouse	VEGF expression, proliferation and vessel count	IGF-IR blockage inhibited HT29 growth in both Monolayer and soft agar ($P < 0.05$). In nude mice it led to ↓ tumour growth ($P < 0.05$)

Lenox or Lubria Broth (LB) Ampicillin Agar medium

10 g NaCl

10 g Tryptone

5g yeast extract

1litre of deionised water

10 ml of 10mg/ml Ampicillin

LB plate

5g NaCl

10g Tryptone

5g yeast

1ml of 1N NaoH

15g agar

1 litre of deionised water

Ampicillin

S.O.C Medium

0.5% Yeast extract,

2.0% tryptone

10mM NaCl

2.5mM KCl

10mM MgCl2

20mM MgSO4

20mM glucose.

1% Acid Alcohol Solution (for differentiation)

Hydrochloric acid ---------- 1 ml

70% ethanol ---------- 100 ml

Mixed well.

0.2% Ammonia Water Solution (Bluing)

Ammonium hydroxide (concentrated) ----- 2 ml

Distilled water ---------- 1000 ml

Mixed well.

Eosin Y Solution:

Eosin Y Stock Solution (1%)

Eosin Y ---------- 10 g

Distilled water ---------- 200 ml

95% Ethanol ---------- 800 ml

Mix to dissolve and store at room temperature.

Eosin Y Working Solution (0.25%)

Eosin Y stock solution ---------- 250 ml

80% Ethanol ---------- 750 ml

Glacial acetic acid (concentrated) ---- 5 ml

Mix well and store at room temperature.

Haematoxylin Solution (Harris)

Potassium or ammonium (alum) ----- 100 g

Distilled water ---------- 1000 ml

The solutions were heated to dissolve. Then 50 ml of 10% alcoholic haematoxylin solution was added and boiled for 1 minute. Then 2.5 g of mercuric oxide (red) was added and heated it until it becomes dark purple colour. The solution was cooled in cold water bath and 20 ml of glacial acetic acid (concentrated) was added. The solution was filtered before use.

Reagents for Electron Microscopy

Glutaraldehyde

(20mls 20% Para formaldehyde [Analar BDH] + 16mls 25% glutaraldehyde [TAAB] + 59mls phosphate buffered saline [Oxoid])

Osmium tetroxide

1% osmium tetroxide [Analar BDH] + 1.5% potassium ferricyanide [BDH] in PBS [Oxoid]

Toluidene blue stain

1% toluidene blue [Raymond Lamb] with 0.2% pyronine [Raymond Lamb] in 1% sodium tetraborate [Analar BDH].

Reynolds lead citrate

Dissolve 1.33g lead nitrate [BDH] in 15mls distilled water and 1.76g sodium citrate [BDH] in 15mls distilled water, mix solutions together and dissolve the resulting precipitate with 8 mls of 1M sodium hydroxide [BDH], make up to final volume of 50mls

Lemix epoxy resin

Lemix A (25mls) + Lemix B (55mls) + Lemix D (20mls). Poured into plastic resin bottle and add 2 mls of BDMA, mixed well.

Thin section were cut with diamond knife and stained with uranyl acetate and lead citrate for examination with an electron microscope. Cubes of tissue less than 2 mm^3 in thickness were cut from the specimen and fixed in 1.5% paraformaldehyde/1.5% glutaraldehyde in phosphate buffer for a minimum of 2 hours. The tissue was then washed with several changes of phosphate buffered saline [Oxoid] and post fixed using 1% osmium tetroxide / 1.5% potassium ferricyanide for 12 hours.

The tumour sections were washed with distilled water and dehydrated using graded alcohols 30%, 50%, 2x70% and 3x100%. They were then kept in a 50% alcohol / 50% Lemix (TAAB) epoxy resin mixture overnight and the following day infiltrated with 100% lemix resin for a minimum of 6 hours. This was followed by embedding them in fresh lemix resin in plastic embedding capsules and polymerised in an oven at 70°C overnight. One μm sections were cut using glass knives on a Reichert-Jung ultra cut microtome, collected on glass microscope slides and stained using 1% Toluidene blue / 0.2% pyronine in 1% Borax stain.

Procedure for electron microscopy

Sixty to ninety nanometre sections were cut using a diamond knife (Diatome) and collected on 300 HS, 3.05 mm copper grids (Gilder). The sections were stained with saturated uranyl acetate in 50% ethanol (TAAB) for 5 mins, washed in a stream of distilled water and then stained with Reynolds' lead citrate for 5 min. The sections were washed again and left to dry before viewing. The ultra thin sections (60-90 nm) were viewed and photographed using a Philips CM120 transmission electron microscope.

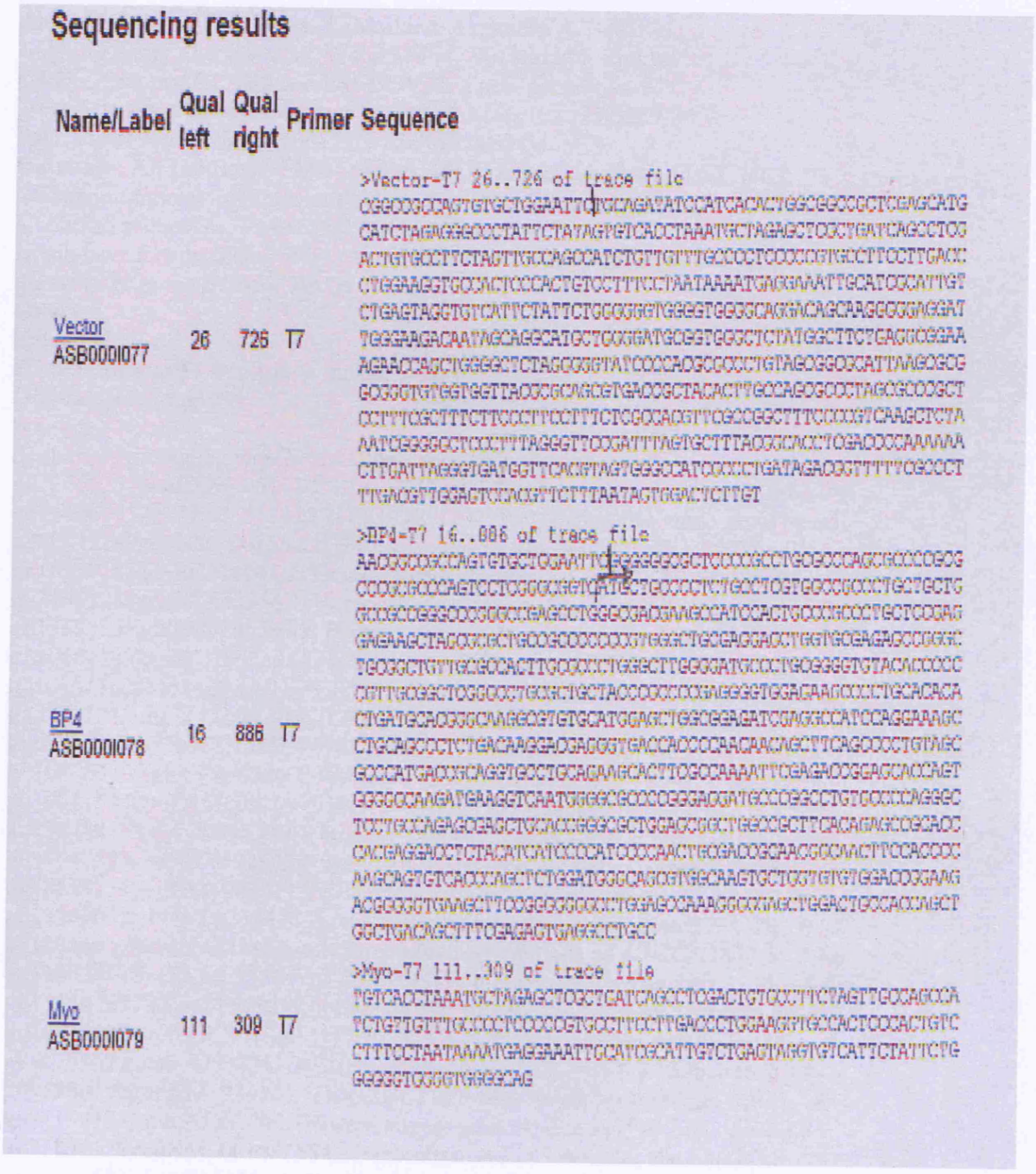


Figure A1 showing the results of sequencing analysis of the plasmids. It confirms the presence of IGFBP-4 cDNA insert and pcDNA3 in the samples.

BLASTN 2.2.15 [Oct-15-2006]

Reference:
Altschul, Stephen F., Thomas L. Madden, Alejandro A. Schäffer,
Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman
(1997), "Gapped BLAST and PSI-BLAST: a new generation of
protein database search programs", Nucleic Acids Res. 25:3389-3402.
RID: 1166190668-28633-200171592568.BLASTQ4
Database: All GenBank+EMBL+DDBJ+PDB sequences (but no EST, STS,
GSS,environmental samples or Phase 0, 1 or 2 HTGS sequences)
4,658,713 sequences; 18,685,068,004 total letters
If you have any problems or questions with the results of this search
please refer to the **BLAST FAQs**
Query=
Length=35
Distribution of 51 Blast Hits on the Query Sequence
Distance tree of results
Score E
Sequences producing significant alignments: (Bits) Value
gi|114667554|ref|XM_001170312.1| PREDICTED: Pan troglodytes s... 52.0 6e-05
gi|114667552|ref|XM_511475.2| PREDICTED: Pan troglodytes simi... 52.0 6e-05
gi|62243289|ref|NM_001552.2| Homo sapiens insulin-like growth... 52.0 6e-05
gi|16359145|gb|BC016041.1| Homo sapiens insulin-like growth f... 52.0 6e-05
gi|37953288|gb|AY442346.1| Homo sapiens insulin-like growth f... 52.0 6e-05
gi|23396306|gb|AC018629.12| Homo sapiens chromosome 17, clone RP 52.0 6e-05
gi|50484189|emb|CR603382.1| full-length cDNA clone CS0DM004YJ... 52.0 6e-05
gi|184815|gb|M62403.1|HUMIGFBP5 Human insulin-like growth fac... 52.0 6e-05
gi|2765191|emb|Y12508.1|HSIGFBP H.sapiens IGFBP-4 gene 52.0 6e-05
gi|695253|gb|U20982.1|HSU20982 Human insulin-like growth fact... 52.0 6e-05
gi|108764099|gb|CP000386.1| Rubrobacter xylanophilus DSM 9941, c 40.1 0.25
gi|109115230|ref|XM_001097914.1| PREDICTED: Macaca mulatta si... 38.2 0.97
gi|19848415|gb|AC009630.14| Homo sapiens chromosome 8, clone RP1 38.2 0.97
gi|39647555|emb|BX572595.1| Rhodopseudomonas palustris CGA009 co 38.2 0.97
gi|58585231|ref|NM_001011556.1| Canis familiaris ceroid-lipof... 38.2 0.97
gi|29294032|gb|AC092384.5| Homo sapiens chromosome 16 clone RP11 38.2 0.97
gi|117164170|emb|AM238664.1| Streptomyces ambofaciens ATCC 23877 36.2 3.8
gi|113428918|ref|XM_933347.2| PREDICTED: Homo sapiens hypothe... 36.2 3.8
gi|111147037|emb|CT573213.2| Frankia alni str. ACN14A chromosome 36.2 3.8
gi|109122324|ref|XR_010720.1| PREDICTED: Macaca mulatta simil... 36.2 3.8
gi|96771622|emb|AJ937741.1| Streptomyces ambofaciens ATCC 238... 36.2 3.8
gi|94386256|ref|XM_984231.1| PREDICTED: Mus musculus hypothet... 36.2 3.8
gi|91199575|emb|AJ937740.1| Streptomyces ambofaciens ATCC 238... 36.2 3.8
gi|71896128|ref|NM_001031584.1| Gallus gallus POU domain, cla... 36.2 3.8
gi|55831587|gb|AC151766.1| Ornithorhynchus anatinus chromosom... 36.2 3.8
gi|33521039|gb|AY280635.1| Uncultured bacterium clone pbiow g... 36.2 3.8
gi|33457241|gb|AC127554.4| Mus musculus BAC clone RP24-323K23... 36.2 3.8
gi|28630143|gb|AC124170.3| Mus musculus BAC clone RP23-155H5 fro 36.2 3.8
Mouse-over to show defline and scores, click to show alignments

(RID=1166190668-28633-200171592568.BLASTQ4, Pagina 1 di 10
http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi 15/12/2006)

Figure A2 showing confirmation of IGFBP-4 cDNA in the gene contruct as per PubMed

database.

Table 1A Animal weight (Late gene transfer model))

Animal	Week 0	Week 1	Week 2	Week 3
M1	31	28	27.3	28
M2	31	31	32	31
M3	28	28	27.3	28
M4	31	31	31	30
M5	29	29	29.3	29
M6	29	29	29.5	29
B1	30	30	29.8	29
B2	31	32	31.2	32
B3	35	36	35.6	35
B4	33	34	34.1	34
B5	26	27	27.1	27
B6	33	33	33	33

Table 1B Animal weight (Early gene transfer model)

week	P1	P2	P3	P4	P5	P6
1	27	28	30	28	30	31
4	29	29	30	30	27	32
	M1	**M2**	**M3**	**M4**	**M5**	**M6**
1	31	31	30	30	30	28
4	31.3	26	30.4	29.5	30.2	29.5
	B1	**B2**	**B3**	**B4**	**B5**	**B6**
1	25	30	29	29	27	29
4	31.5	30	31	29.5	30	29.5

Key: B1-B6 = BP-4 group, M1-M6= Control plasmid group, P1-6 Control group without any plasmid; 0, 1, 2, 3 and 4 = 0, 1, 2, 3 and 4 weeks following gene therapy.

Table 2A Tumour size (Late gene transfer model)

Animal	Week 0 (LXB)	Week 1	Week 2	Week 3 (LXBXW)
M1	6X5.5	8X8.3	12.8X10.1	14.1X9.1X9.1
M2	7.3X6.4	10.6X6.2	15.4X11.8	20X11.1X7
M3	7.4X4.4	10.4X5.8	12.8X8.1	12.3X7.5X6.8
M4	5.2X5.1	11X8.1	11.8X9.6	12.3X9X5.4
M5	9.6X4.6	13.5X10.5	17.7X10.8	17.5X11.5X11.5
M6	2.2X2	7.8X3.9	10.5X9.4	13.2X7.2X4.7
B1	5.8X3.6	7.4X7.4	14X8.7	12.5X7.5X5.7
B2	9.9X4.6	12.4X11.7	15.9X14.8	14.4X12.6X14.6
B3	9.4X6	12.1X12.1	16.1X14.5	16.6X14.9X5.9
B4	5.8X5.3	9.5X9.5	15X12.9	15X13.2X6.5
B5	8.5X5.8	10.6X8.5	14.9X9.7	16X12X9
B6	11.2X7.5	15.8X10.3	13.4X11.2	17X13X9

Table 2B Tumour volume (mm^3) (Late gene transfer model)

Animal	Week 0	Week 1	Week 2	Week 3
M1	99	275	827	917
M2	170	348	1399	984
M3	120	313	663	492
M4	69	490	668	470
M5	211	956	1691	1153
M6	5	118	518	350
B1	265	885	1879	1145
B2	60	202	852	420
B3	89	428	1451	1010
B4	190	477	1076	1356
B5	470	1285	1005	1561
B6	225	899	1870	2080

Key: B1-B6 = BP-4 group, M1-M6= Control plasmid group

0, 1, 2 and 3 = 0, 1, 2 and 3 weeks following gene therapy

Table 3A Tumour size in the early gene transfer group

week	P1 (LXB)	P2	P3	P4	P5	P6
1	12.2X7.3	10.2X8.8	12.9X8.1	9X8	9X9	8.4X6.6
4	18.5x16.6	22.8x18	20.6x12.9	16.4x15.1	15.4x12.6	15.3x13.1
	M1	M2	M3	M4	M5	M6
1	7.2X7.2	11X8.5	11.1X8.6	7.3X7.3	8.8X5.6	8.5x8.3
4	10.9x10.9	15.6x14.8	11.3x7.8	14x11	16.1x8.1	13x11.8
	B1	B2	B3	B4	B5	B6
1	7.4X7.1	7.8X6.5	7.5X6.7	7.2X7.2	7X4.5	8.1X8.1
4	13x11.1	17.5x13.5	12.7x7.4	11.1x10.8	15x10	17.4x14.8

Table 3B Tumour volume in the early gene transfer group

Week	P1	P2	P3	P4	P5	P6
1	325.069	394.944	423.184	288	364.5	182.952
4	2548.93	3693.6	1714.02	1869.68	1222.45	1312.82
	M1	M2	M3	M4	M5	M6
1	186.517	165.775	168.338	74.925	186.624	265.721
4	1708.51	647.515	343.746	528.16	847	905.06
	B1	B2	B3	B4	B5	B6
1	186.624	397.375	410.478	210.937	137.984	292.782
4	800.865	1594.69	347.726	647.352	750	1905.65

Key: P1-6 = Plain group, M1-6 = Control plasmid group, B1-6 = BP-4 group

1 and 4 = 1 and 4 weeks after plasmid therapy

Table 4A Mitotic figures in the late gene transfer model

M1	M2	M3	M4	M5	M6	B1	B2	B3	B4	B5	B6
6	0	4	7	6	1	3	1	2	2	1	0
3	0	10	2	3	5	6	3	8	4	5	2
4	4	2	5	7	5	4	3	3	2	3	2
2	2	4	3	3	3	2	0	2	2	0	1
2	3	4	1	7	2	5	0	5	0	2	4
2	0	4	0	10	1	3	0	3	2	6	0
5	2	2	6	8	4	3	0	3	3	2	2
3	2	4	11	7	3	4	0	7	3	0	2
1	3	2	2	5	1	1	0	6	2	1	1
2	1	2	5	7	2	2	1	2	1	0	2

Table 4B Mitotic figures in the early gene transfer model

B1	B2	B3	B4	B5	B6	M1	M2	M3
10	5	2	5	1	0	0	2	2
1	4	1	0	1	1	1	0	2
9	2	1	0	2	2	1	4	1
2	0	2	2	1	1	0	2	0
0	2	1	2	0	1	1	1	2
M4	**M5**	**M6**	**P1**	**P2**	**P3**	**P4**	**P5**	**P6**
14	3	9	6	8	5	20	5	7
7	8	4	1	8	8	9	5	8
1	5	4	4	11	15	10	32	6
13	1	5	6	4	1	17	2	3
1	0	0	0	0	2	0	4	3

Key: C1-6 = Control plasmid group, B1-6 = BP-4 group P1-6= Control no plasmid group

Table 5A Cell death score in late gene transfer model

B1	B2	B3	B4	B5	B6
2	4	3	2	3	3
0	2	3	3	1	4
2	3	1	3	0	3
1	2	2	4	2	3
1	4	1	2	4	2
M1	**M2**	**M3**	**M4**	**M5**	**M6**
1	0	0	1	2	1
3	3	2	1	0	0
2	2	1	1	0	1
2	2	2	2	1	0
1	3	1	3	0	2

Table 5B Cell death score in early gene transfer model

B1	B2	B3	B4	B5	B6	M1	M2	M3
2	1	3	3	1	5	1	1	2
2	2	3	1	1	4	2	1	1
1	2	1	0	0	4	1	3	4
1	2	1	3	5	3	1	1	2
5	4	2	1	0	3	3	1	4
M4	**M5**	**M6**	**P1**	**P2**	**P3**	**P4**	**P5**	**P6**
3	2	5	0	0	1	3	0	2
1	1	0	0	1	1	0	1	2
3	0	0	0	0	0	1	0	2
0	3	2	1	0	0	0	0	0
0	4	1	2	3	0	0	0	0

Key: P1-6 = plain group, M1-6 = Control plasmid group,

B1-6 = BP-4 group

Table 6A Western blot densitometry for IGFBP-4 (EGTM)

P1	P2	P3	P4	P5	P6
1.181	1.097	0.968	1.25	0.917	0.873
M1	**M2**	**M3**	**M4**	**M5**	**M6**
0.74	0.533	0.674	0.674	0.714	1.214
B1	**B2**	**B3**	**B4**	**B5**	**B6**
0.538	0.594	0.594	0.646	0.676	0.789

Table 6B Western blot densitometry for IGF-IR (LGTM)

Group	Control M	BP-4
1	3.85	4.955
2	4.751	4.313
3	3.573	5.823
4	4.434	5.101
5	2.69	4.843
6	3.138	5.484

Table 6C Western blot densitometry for Bax (EGTM)

P1	P2	P3	P4	P5	P6
1.708	1.484	9.057	2.984	2.622	2.383
M1	**M2**	**M3**	**M4**	**M5**	**M6**
2.829	4.757	1.838	10.225	1.728	3.327
B1	**B2**	**B3**	**B4**	**B5**	**B6**
8.415	8.922	6.986	8.082	2.97	2.495

Table 6D Western blot densitometry for Bcl-2 (EGTM)

P1	P2	P3	P4	P5	P6
1.082	0.944	2.163	5.106	1.248	3.081
M1	**M2**	**M3**	**M4**	**M5**	**M6**
1.421	0.731	0.511	0.541	0.814	2.091
B1	**B2**	**B3**	**B4**	**B5**	**B6**
7.762	12.467	7.109	6.76	1.495	0.779

Key: P1-6 = plain group, M1-6 = Control plasmid group,

B1-6 = BP-4 group

Table 7A Western blot densitometry for Bax (LGTM)

B1	**B2**	**B3**	**B4**	**B5**	**B6**
4.516	3.613	7.451	5.927	19.757	11.29
3.951	3.387	6.492	6.943	16.003	13.675
M1	**M2**	**M3**	**M4**	**M5**	**M6**
7.79	5.588	5.927	5.588	4.572	4.064
5.779	5.08	5.786	5.483	4.191	2.406

Key: B1-B6 = BP-4 group, M1-M6= Control plasmid group

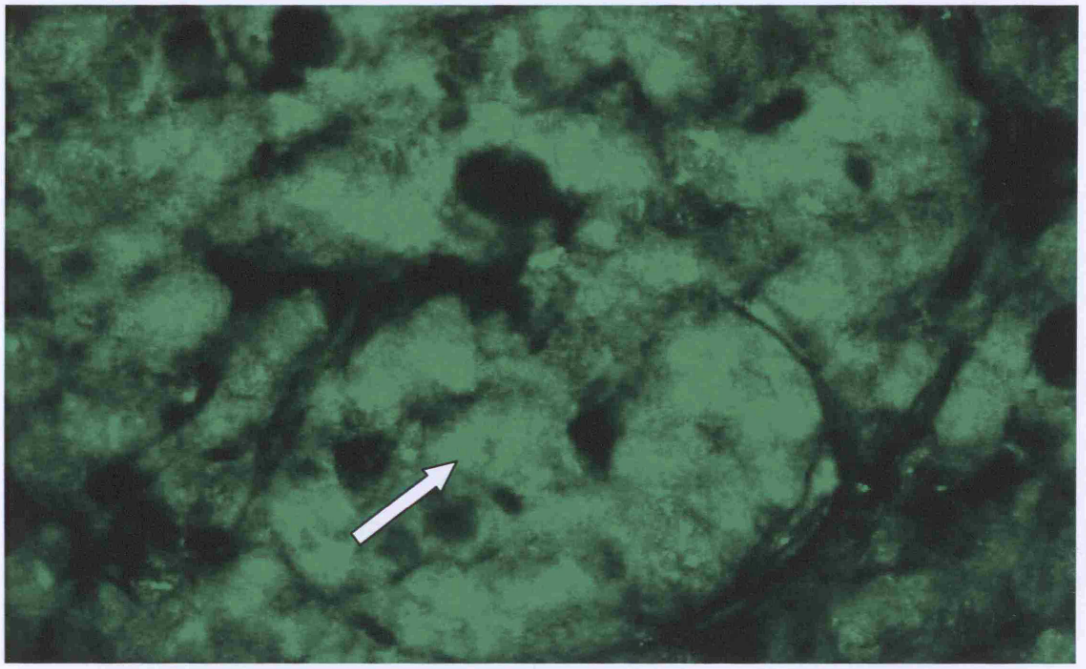


Figure A3 BP-4 immunofluorescence in tumours of BP-4 group (×400) following single administration of BP-4 gene construct in a previously established subcutaneous colon cancer. Greenish immunofluorescence (arrow) from the IGFBP-4 protein is found inside the colon cancer cells.

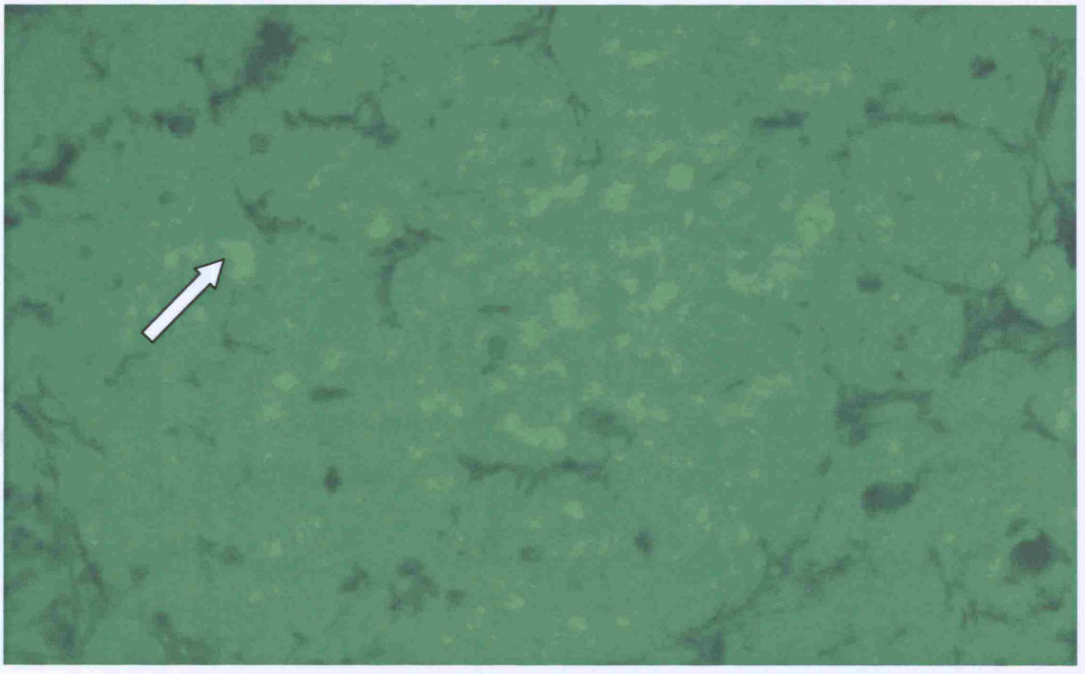


Figure A4 BP-4 treated tumour tissue stained for BP-4 immunofluorescence in the late gene transfer model after single administration of BP-4 gene in a previously established colon cancer (×200). Intracellular IGFBP-4 protein is seen as greenish immunofluorescence (arrow).

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Publications associated with this thesis

1) The role of the insulin-like growth factor system in colorectal cancer: review of current knowledge - **review – International Journal of colorectal disease.** 2005 May; 20(3):203-20

2) Biology of IGFBP-4 and its role in cancer- **review. International Journal of Oncology** 2006 Jun;28(6):1317-25

3) Gene therapy in colon cancer –**review**- Submitted to **Clinical Colorectal Cancer**

4) Increased Apoptosis and Decreased Proliferation of Colorectal Cancer Cells Using Insulin-Like Growth Factor Binding Protein-4 Gene Delivered Locally by Gene Transfer – **research paper Colorectal disease** 2007 Sep;9(7):625-31.

5) Insulin-like growth factor binding protein-4 gene therapy increases apoptosis by altering Bcl-2 and Bax proteins and decreases angiogenesis in colorectal cancer - **research paper - International Journal of Oncology** 2007 Apr;30(4):883-8

6) Role of insulin-like growth factor binding protein-4 in prevention of colon cancer **research paper** Nov 2007 **World Journal of Surgical Oncology**

Presentations associated with this thesis

1) Effect of Insulin-Like Growth Factor Binding Protein-4 plasmid therapy in colon cancer –**Poster**, March 2005 University College London

2) IGFBP-4 gene therapy increases apoptosis of colorectal cancer by altering Bax/Bcl-2 levels. **Oral presentation. SARS** Jan 2006

3) Effect of IGFBP-4 gene therapy on tumour morphology and angiogenesis in colon cancer - **Oral presentation BASO** Nov 2005